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THE IMPORTANCE OF MOLECULAR STRUCTURE IN WETTING AGENTS

BY LEE LEISERSON,\* R. W. BOST AND F. K. CAMERON

*Contribution from the Venable Chemical Laboratory of the University of North Carolina*

It is a well established rule that the incorporation of a slightly soluble substance or an insoluble substance in a liquid results in a segregation of the solute so that the surface layer is more concentrated with respect to the solute than the interior of the liquid. By virtue of the fact that the molecules or ultimate particles of the solute now form part of the surface layer, the surface tension, interfacial tension, or surface energy is lower than those of the pure liquid, the surface viscosity (with its inverse fluidity) and perhaps other properties are altered. A judicious selection of a solute or solutes controls these properties and the resulting characteristics of the solution which make them a penetrant, a wetting agent, a detergent, etc. More particularly, we are concerned with the ability of a liquid to adhere to a solid, i.e., to wet it.

A WETTING AGENT

There are many solutes which are known to increase wetting power, especially in the case of water. The term *wetting agent* is restricted, however, to solutes which are effective in relatively small quantities, hence are cheap to use, and which gather at an interface and are adsorbed quickly, for speed of action is the economic justification of wetting agents in industrial practice. The stresses of adsorption on the wetted surface and of solution in the surface of the wetting liquid must not be too diverse to avoid a tendency of the solute to migrate too much to one of the phases and diminish the effect on wetting. The substances which have been found to meet these requirements have, generally, a more or less complex molecular structure containing both hydrophylic and hydrophobic groups (12). This "duplex" character may be secured by the inclusion of a polar grouping in an otherwise non-polar molecule, or, by inclusion of groups containing N, O, S, or double or triple bonds (interrupting groups) which tend to orient themselves toward the interior of a polar liquid such as

\* Virginia Smelting Company Fellow, 1939-41

water (7). Orientation of the solute in the interface is probably characteristic for all substances. It is certainly the case with wetting agents.

#### ADSORPTION

The phenomena of adsorption have been studied comprehensively and certain governing rules formulated (8). Commonly, it takes place quickly, although there are recorded cases, particularly adsorption from very dilute solutions when the time interval for equilibrium was days, rather than minutes (1).

#### THE SOLUTION

If the solute be of the polar-non-polar type, complete electrolytic dissociation may be assumed at very high dilutions. This assumption appears unreasonable, however, at the concentration of the surface film, where the solution is near saturation. It is more probable that the hydrophobic groupings and perhaps some entire molecules are dispersed as a colloid, and the interface affected accordingly. For instance, pectization, increasing the size but decreasing the number of hydrophobic particles, materially changes the adsorption characteristics of the solute at the interface. It may mean an actual increase of effective particles by closer spacing. This does not imply that "crowding" would be desirable even if we knew how to effect it. Support of this concept comes from the observations of McBain on solutions of certain soaps and his definition of a colloidal electrolyte as one "in which one ion has been replaced by a heavily hydrated polyvalent micelle carrying an equivalent sum total of electrical charges" (10). The concept finds further support from a number of observations that the amount of hydrophobic material in the interface is much in excess of that indicated by theoretical formulas (Bancroft) (3). Many well known dyes are colloids.

It may be true that there is no causal connection between adsorption and surface tension. The latter is dominant, however, in determining the interfacial tension or its numerical equivalent, the surface energy. Because of experimental difficulties, neither the classical methods of Young (14) nor Gibbs (6) nor the more recent approach of Bartell and colleagues (4) suffice for testing purposes. Moreover they are not applicable to cases where the solute is a colloid. It is true we can now obtain the capillary angle with fair accuracy (4.9) also the surface tension in the liquid-gas interface; but not as yet have we a satisfactory method for determining the surface tension of the solid phase in contact with either liquid or gas. We must content ourselves in knowing the interfacial tension must be low for good wetting.

#### RATE OF WETTING

We cannot, therefore, employ the determination of the interfacial tension as a practical control in wetting processes. To this end a technique has been developed to observe the rate at which a standard skein of cotton (2) or a standard disk of canvas (11) sinks through the solution. The observations are in accord with the equation  $t^n = kc$ , where  $t$  is the sinking time for a given dis-

tance,  $c$  is the concentration and  $n$  and  $k$  are constants. In logarithmic form the equation is linear. It is simpler, in practice, to plot the observations on logarithmic scaled paper. The constant  $n$  is then the slope of the curve obtained, but there is no hint of its physical significance, if any.

#### MOLECULAR STRUCTURE

The molecular structure of a wetting agent is of primary importance. According to Traube's rule adsorption increases with the number of carbon atoms. But there is a critical limit and this has been studied for special cases (1). It is desirable to have a high mobility in the liquid interface and to this end a lower sum of inner molecular stresses than the solution tension which is itself low for a slightly soluble substance. It is desirable to have as wide a covering as possible of the area in the interface, with hydrophobic groups. To this end, it is desirable to have the polar or the interrupting grouping midway in a chain compound or in an ortho position in an aromatic compound. The protrusion of the greatest number possible of hydrophobic groups will favor an interface of low surface energy necessary to effective wetting.

A desirable group content of the hydrophobic portion of the molecule is suggested in British patents Nos. 479835 and 479897 issued to I. G. Farbenindustrie in 1938. Adding to this group formula the symbol  $W$  for a solubilizing group or groups and generalizing to a greater extent one may write an "ideal" formula:



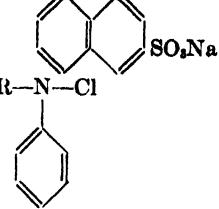
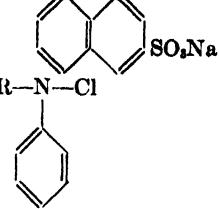
wherein  $A$  represents a number of carbon atoms, as an alkyl group,  $n$  the number of times the alkyl radical appears on a nucleus which may be aromatic, hydroaromatic or heterocyclic;  $B$  represents an interrupting group such as  $-O-$ ,  $NH-$ ,  $-C\equiv C-$ , etc.;  $C$  represents a short hydrocarbon chain, and  $W$  represents a hydrophylic group which may be attached anywhere in the molecule but preferentially, for high wetting activity, near the center. Several of the features suggested by this formula but not necessarily all of them are to be found in any of the generally accepted wetting agents.

#### WETTING BY COMMERCIAL REAGENTS

Sinking times for several concentrations were made for each of a number of commercial surface active preparations. Each solution tested was of 500 cc.'s volume and in preparing it from a liquid reagent, one cubic centimeter of the reagent was assumed to weigh a gram, obviously incorrect, but a sufficient approximation for present purposes. The method of Seyferth and Morgan (11) was followed as more convenient than that proposed by Draves (2). The disks, one inch in diameter, were cut from the same piece of canvas. Each sinking time recorded was the average of at least 3 closely agreeing observations. For each reagent, when sinking time against concentration was plotted on logarithmic scaled paper a straight line was obtained. With possibly 3 exceptions these lines approached parallelism, suggesting that the assumed con-

stant  $n$  is largely, if not completely, independent of the nature of the particular reagent. If this be so, it seems unlikely that a diluent would be responsible for

TABLE I  
*General Formulae for Surface Active Compounds*

TYPE	GENERAL FORMULA	NAME
A 1	$\text{RCOONa}$	Salt of a fatty acid
2.	$\text{C}_n(\text{H}_{2n-2})(\text{OSO}_3\text{Na})\text{COONa}$	Disodium salt of sulfated fatty acid
3	$\text{RCOOCH}_2\text{CHOHCH}_2\text{OH}$	Glyceryl ester of fatty acid—sometimes sulfated
4	$\text{RCOOCH}_2\text{CH}_2\text{SO}_3\text{Na}$	Sodium fatty acid ester sulfonate
5	$\text{RCONHCH}_2\text{CH}_2\text{SO}_3\text{Na}$	Sodium fatty acid amide sulfonate
6.	$\text{RCONHCH}_2\text{CH}_2\text{NR}_2$	Fatty acid amido dialkyl amine
7	$\text{R—O}(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CH}_2\text{OH}$	Polyether alcohol
B 1	$\text{ROSO}_3\text{Na}$	Sodium alkyl sulfate, R is large
2	$\text{R}_2\text{CHOSO}_3\text{Na}$	Sodium secondary alkyl sulfate
3	$\text{ROOC—CH}_2$   $\text{ROOC—CHOSO}_3\text{Na}$	
C 1.	$\text{RSO}_3\text{Na}$	Sodium alkyl sulfate, of a dialkyl carboxylate
2	$\text{R—Aryl—SO}_3\text{Na}$	Sodium alkyl aryl sulfonate
3	$\text{R}_2\text{Aryl—SO}_3\text{Na}$	Sodium dialkyl aryl sulfonate
4	$\text{ROOC—CH}$   $\text{ROOC—CHHSO}_3\text{Na}$	Sodium salt of the sulfonic acid derivative of a dialkyl carboxylate
5	$\text{CH}_3(\text{CH}_2)_7\text{CHCH}_2(\text{CH}_2)_7\text{COONa}$ 	Sodium salt of oleic acid naphthalene sulfonic acid condensation product
D 1		Alkyl pyridinium salt where R is an aliphatic chain
2	$\text{RR}'\text{R}''\text{N—Cl}$	Alkyl alkyl aryl alkyl quaternary ammonium salt
E 1	$\text{ROH}$	Alcohol or phenol
2	$\text{RNH}_2$	Amine
3	$\text{RR}'\text{CO}$	Ketone
4	$\text{RCOOH}$	Carboxylic acid
5	$\text{RR}'$	Hydrocarbon

*Note:* The salts shown are merely illustrative. The acids may be neutralized with one of a wide variety of bases. Similarly, the substituted ammonia compounds may be neutralized with one of a large number of acids.

the deviations in the slope. The deviations cannot be attributed satisfactorily to the idiosyncrasies of the surfaces of the individual canvas disks, for in that case the several points could not be expected to fall on a straight line. As yet we have no inkling of a physical significance for  $n$  nor upon what it depends.

From interpolation of the several curves we can obtain the weights of reagents per liter of solution to cause sinking for any particular time. As the commercial purpose in the use of a wetting agent is to effect rapid wetting, we have chosen 30 seconds. There can be no pretension of absolute values for these figures, but it is thought they do serve to show the relative values as wetting agents of the reagents named in so far as they were correctly represented by the samples at hand. The results are assembled in Table II according to the type designa-

TABLE II  
*Comparative Efficiency of Various Wetting Agents\**

SUBSTANCE	TYPE	CONCENTRATION OF WETTING AGENT GRAMS PER LITER IF SOLID, CC. PER LITER IF LIQUID
Aerosol OT Dry	C 4	0.205 g.
Santomerse D	C 3	0.66 g.
Tergitol 7	B 2	0.69 cc
Sodium Oleate	A 1	0.80 g.
M P 189	C 3	1.06 g
Triton NE	A 7	1.20 cc
Zephiran	D 2	1.20 g
Artic Syntex T	A 5	1.47 g
Alkanol B	C 3	1.50 g.
Triton E 79	B 1 (Modified)	1.55 cc
Igepon AP Extra†	A 4	1.57 g.
Deceresol OT Clear 10%	C 4	2.00 cc.
T L F 122		2.03 cc.
Wetanol	A 2 (Modified)	2.33 g.
Triton 720	C 2 (Modified)	2.55 cc.
70% Sodium Glycocholate (Crude)	A 5	2.85 g.
Deceresol OS	C 2	2.95 g.
Triton W 30	B 1 (Modified)	3.31 cc.
Deceresol OT Aqueous 10%	C 4	3.95 cc.
Nekal A	C 2	4.30 g.
Igepon T Gelt†	A 5	5.25 g.
85% Turkey Red Oil†	A 2	11.0 cc.

\* Thirty second sinking time of standard canvas disk at 25°C.

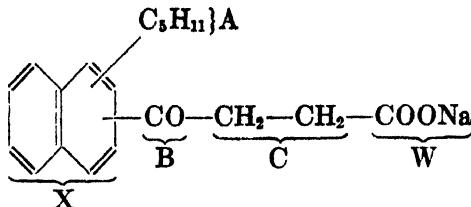
† These values are not a true indication of wetting efficiency for the slope of the curves differs radically from that of the other substances.

tion that is given in Table I. The authors have combined information from tables by Degering (5) and Van Antwerpen (13) to produce an enlarged table in the form proposed by Degering.

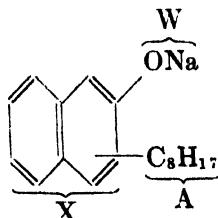
#### WETTING AGENTS

It would appear from an inspection of the formulas of the supposedly active substances in the reagents cited in Table I that the nearer the formula approached the "ideal formula,"  $(A)_n \cdot X \cdot B \cdot C \cdot W$ ., the more assured is the wetting activity of the substance. It is clearly desirable to check this thesis by tests with substances that were not the usual commercial mixtures. For this purpose several compounds were synthesized.

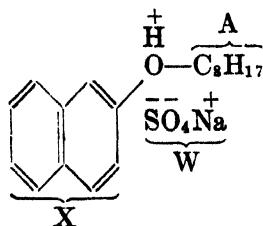
1. It was found that sodium  $\beta$ -(amylnaphthoyl) propionate which conforms to the formula shown below is very active and an excellent wetting agent but is precipitated by acids. A solution containing 0.8 gram per liter sinks a canvas disk in 30 seconds.



2. A solution containing 1 gram isooctyl  $\beta$ -naphthol, 4 grams sodium hydroxide, and 50 cc. of alcohol per liter causes a canvas disk to sink almost instantaneously.



3. In the formation of isooctyl  $\beta$ -naphthol by the condensation of diisobutylene and  $\beta$ -naphthol in the presence of sulfuric acid a condensation product is formed. This material, probably sodium isooctyl  $\beta$ -naphthyl ether oxonium sulfate, is an effective wetting agent in both acid and alkaline solutions. A concentration of 1.8 grams per liter causes the canvas disk to sink in 30 seconds.



The observations on these substances confirm the ideas advanced above, that the presence of hydrophobic groupings together with hydrophilic groupings in a molecule make for wetting activity. They indicate also, however, that the presence of these groupings is not in itself a guarantee of effectiveness, but there must be a suitable arrangement of groupings, perhaps involving a balancing of internal stresses. In other words, the molecular structure is the dominant factor in determining effectiveness in a wetting agent.

## CONCLUSIONS

From the foregoing discussion and the experimental results recorded, the following conclusions may be drawn:

1. In the interface, it may be assumed that the hydrophobic portions of the molecules of a wetting agent are dispersed as a colloid, although the hydrophilic grouping may be in true solution.
2. The effectiveness of a wetting agent is determined by its molecular structure.
3. The position and arrangement of groups in a molecule are no less important than their presence, in determining the effectiveness of a wetting agent.

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## SOME NEW DERIVATIVES OF NICOTINE

By A. L. ALEXANDER AND R. W. BOST

*Contribution from the Venable Chemical Laboratory of the University of North Carolina*

In the manufacture of tobacco products there is a considerable waste of material in the form of stems and other fibrous constituents. Some of the poorer grades lose as much as thirty-three per cent by weight during the stemming process. The average waste in the manufacture of tobacco has been conservatively estimated at twenty per cent. In order to obtain an idea of the magnitude of loss during manufacture, the amount of tobacco produced throughout the world in 1938 (this is the last year complete data are available) was 6,167,518,000 pounds (1). The 1938 loss calculated on the basis of twenty per cent amounted to 1,233,503,600 pounds. On the same basis, the waste products from the 1,385,573,000 pounds (2) of tobacco produced in the United States during the same year amounted to 277,114,600 pounds. In 1938 North Carolina produced 517,210,000 pounds (3) of tobacco, which calculated on the basis of twenty per cent loss gave 103,442,000 pounds.

Aside from the aforementioned loss on harvested tobacco, there remains in the field the stalks which are unfit for sale. At present no use is made of them other than for fertilizer. No data are available with regard to the number of pounds represented by this material.

In view of the large supply of waste products which the tobacco plant affords, it seemed desirable to make a study to determine if these products could be utilized to a fuller extent in the chemical field. At the present time part of the stems and fibrous constituents are processed for their nicotine content.

Nicotine is perhaps the most important chemical compound occurring in the tobacco plant. The nicotine content varies with different tobaccos from 0.4 to 4 per cent (some tobaccos have been reported to contain as high as 8% of nicotine). Assuming the loss during the manufacture to be 20 per cent and the nicotine content of the waste products to be one per cent, the nicotine obtainable from the 1938 tobacco crop grown in North Carolina amounted to 1,034,420 pounds. This does not take into consideration the waste represented by the stalks left in the fields after harvesting. The chief uses of nicotine at present are as an insecticide, and in the manufacture of nicotinic acid and nicotinamide.

The purpose of this investigation was to extend the use of nicotine to the production of new compounds which might possess antipyretic, analgesic and local anesthetic properties. Accordingly, nicotine has been converted into nicotinic acid and thence into nicotinyl chloride hydrochloride. Methods have been worked out for the synthesis of the latter compound in a high state of purity. It has been studied with various amines, amino alcohols, and phenols. Attempts to obtain a definite product with salicylic acid were unsuccess-

ful. In the following paragraphs are described the preparation of certain derivatives of nicotinyl chloride hydrochloride.

#### EXPERIMENTAL

*Nicotinic Acid.* This acid was prepared by the oxidation of nicotine employing the method of McElvain (4).

*Nicotinyl Chloride.* The method of Ingersoll and Robbins (5) was used to prepare the acid chloride by the action of thionyl chloride on nicotinic acid.

*Nicotinyl Chloride Hydrochloride.* This compound was prepared by a modification of the method of Spath and Spitzer (6). A mixture of 10 g. of dry nicotinic acid and 65 cc. of thionyl chloride were refluxed for two hours at which time hydrogen chloride and sulfur dioxide were no longer evolved. The solution was then transferred to a sublimation apparatus consisting of a large test tube of about 500 cc. capacity attached to a condenser provided with a receiver suitable for vacuum distillation. An oil bath was used as a heating medium. The excess thionyl chloride was distilled at atmospheric pressure. This was found to prevent loss of the nicotinyl chloride hydrochloride. However, reduced pressure was applied just before the last trace of the solvent was removed. It was found that unless all traces of thionyl chloride were removed the final product was colored yellow. The temperature of the oil bath was not allowed to go above 120° until all of the thionyl chloride had been expelled. At approximately 140° beautiful snow white crystals began to appear on the upper end of the sublimation tube. The temperature during sublimation was never allowed to go above 160°. The yield was 64.4% and the product melted at 149–150°. Analysis for chlorine: Calculated, 39.88%; Found, 39.86%.

*Nicotinanilide.* To 3.5 g. of pure nicotinyl chloride hydrochloride in a small round bottom flask was slowly added 25 cc. of aniline. A vigorous reaction took place with the evolution of much heat. After heating for five minutes, the contents of the flask were washed into a beaker with cold water, filtered, and the filter washed with cold water made faintly alkaline with dilute ammonium hydroxide to remove the excess aniline. The crude nicotinanilide melted at 77–78°. After purification through its hydrochloride in the conventional manner, it melted at 82°. Yield of pure product, 55%. The hydrochloride melted at 202°. It was prepared in the usual way by passing hydrogen chloride into an ether solution of nicotinanilide. Analysis of nicotinanilide hydrochloride for chlorine: Calculated, 15.15%; Found, 15.01%.

*Nicotinyl Phenetidide.* This compound was prepared by the same method as that employed to prepare nicotinanilide except phenetidine was used instead of aniline. The crude product was purified through its hydrochloride until a constant melting point was obtained. M.P. 171°. Yield 60% of theoretical.

The nicotinyl phenetidide hydrochloride was prepared by suspending the nicotinyl phenetidide in 100 cc. of dry ether and adding absolute alcohol until complete solution took place—the nicotinyl phenetidide being difficultly soluble in ether. The solution was then saturated with hydrogen chloride, the hydro-

chloride filtered off and dried in a vacuum desiccator. M.P. 206°. Analysis for chlorine: Calculated, 12.75%; Found 12.64%.

*Phenyl Nicotinate.* A mixture of 5 g. of nicotinyl chloride hydrochloride and 10 g. of phenol were heated under reflux until no more hydrogen chloride was evolved. The reaction product was dumped into a beaker of cold water and made strongly acidic with hydrochloric acid and then extracted six times with ether to remove the excess phenol. The aqueous portion was made alkaline with ammonium hydroxide, whereupon a deep yellow oil separated to the bottom. The solution was saturated with salt, extracted six times with ether and the ether extract dried over sodium sulfate, after which hydrogen chloride was introduced to precipitate the phenyl nicotinate hydrochloride. Yield 61% of theoretical. M.P. 186°. Analysis for chlorine: Calculated 15.09%; Found 15.15%.

Some of the phenyl nicotinate hydrochloride was dissolved in water and the free base precipitated with dilute NaOH. The phenyl nicotinate separated as white crystalline needles melting at 74°.

*Diethylaminoethyl Nicotinate.* 3.5 g. of pure nicotinyl chloride hydrochloride were placed in a 500 cc., 3 neck flask connected with a reflux condenser and covered with 500 cc. of anhydrous acetone. 2.5 g. of diethylaminoethanol, dissolved in 15 cc. of acetone, was added dropwise with constant stirring.

Reaction took place as soon as the alcohol came into contact with the acid chloride. The reaction mixture was stirred and refluxed for two hours, then allowed to cool, and the finely divided precipitate filtered and dried in a desiccator. Yield 65% of the theoretical.

The product was purified by sublimation in an atmosphere of dry hydrogen chloride at approximately 25 mm. pressure. After two sublimations the melting point remained constant at 237°. Analysis for chlorine: Calculated, 24.07%, Found 24.00%. The analyses indicate that the compound is the dihydrochloride of diethylaminoethyl nicotinate. It is very soluble in water. Its aqueous solution, when neutralized with NaOH, possesses the distinct odor of the original amino-alcohol indicating that hydrolysis had taken place. Preliminary studies have shown that the substance possesses local anesthetic action. Further work on its pharmacological properties will be undertaken and reported later.

#### SUMMARY

1. It has been pointed out that tobacco affords an abundant supply of waste material in the form of stems and stalks worthy of chemical study.
2. At the present time nicotine is the chief chemical compound obtained from tobacco.
3. The amount of nicotine derivable from waste tobacco is enormous.
4. An improved method has been worked out for the synthesis and purification of nicotinyl chloride hydrochloride from nicotine.
5. The preparation of a number of new derivatives of nicotine has been described.
6. Their pharmacological properties will be studied and reported elsewhere.

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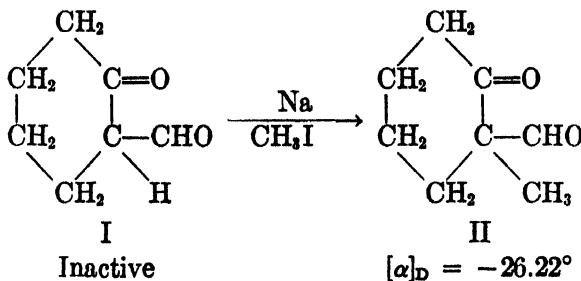
# AN ATTEMPT TO REPEAT A REPORTED ASYMMETRIC SYNTHESIS

BY ARTHUR ROE AND ROBERT L. MCKEE

*Contribution from the Venable Chemical Laboratory of the University of North Carolina*

Recently there appeared in the literature an article claiming a heretofore impossible achievement—the preparation of an optically active compound from an optically inactive compound without the aid of asymmetric carbon atoms, polarized light, or other asymmetric conditions. Such a total asymmetric synthesis is of great theoretical interest, as the problem of the mechanism of formation of the first optically active substance in the universe has not been satisfactorily solved.

This extraordinary claim was made by four Indian chemists (1) who developed a synthesis of santonin. They first reported that the synthetic product was optically inactive. In a later report (2), however, they stated that the synthetic santonin was not the racemate, as had been supposed, but rather was optically active, having a rotation of  $[\alpha]_D = -150^\circ$ . In an attempt to discover the step in which the asymmetric synthesis had taken place the work was repeated. The intermediates were too highly colored, however, to allow a reading in the polarimeter. The same reactions were then carried out on analogous compounds of simpler structure. In one of these experiments it was found that whereas 2-formylcyclohexanone (I) was optically inactive, the crude 2-methyl-2-formylcyclohexanone (II) prepared from it had a rotation of  $[\alpha]_D = -26.22^\circ$ .



We have repeated the above experiment in an effort to duplicate this remarkable synthesis; two different samples of 2-methyl-2-formylcyclohexanone (II) were prepared. Both samples were examined in the polarimeter; no trace whatever of optical activity was observed. After this work was completed O'Gorman (3) and Cornforth, Cornforth, and Dewar (4) reported that they, too, failed to observe optical activity in this same compound prepared by them.

It has been calculated by Cornforth, Cornforth, and Dewar (4) that the probability of accomplishing an asymmetric synthesis is one in about  $10^{200}$ . If the Indian chemists did indeed achieve an asymmetric synthesis it is highly improbable that such a feat could be duplicated. The achievement of our

Indian colleagues is the more remarkable when it is realized that they performed not one but four different asymmetric syntheses—santonin itself, and the three simpler analogous compounds of which only one is discussed in this paper.

#### EXPERIMENTAL

*2-Formylcyclohexanone* (1). A 250 cc. 3-neck flask fitted with stirrer, condenser, and addition funnel, was used. 5.75 g. (0.25 mole) of sodium was dissolved in 125 cc. of absolute methanol. After cooling, 5 g. of methyl formate was added to the solution. The remainder of the methyl formate (total = 15 g., 0.25 mole) was mixed with 24.5 g. (0.25 mole) of cyclohexanone. This mixture was added dropwise to the flask, the addition taking 1½ hours. No temperature rise was observed; the reaction stood overnight.

The reaction mixture was removed to a Claisen flask and the methanol removed at 40° and 25 mm. pressure. The white residue was dissolved in 175 cc. of water and extracted three times with 50 cc. of ether to remove any unreacted starting materials. The aqueous solution was then neutralized with glacial acetic acid, whereupon about 15 cc. of a yellowish oil separated. The solution was extracted thrice with 40 cc. of ether, the ether extracts combined, dried, and the solvent removed under reduced pressure. The residue was distilled, 12 g. of 2-formylcyclohexanone distilling at 104°/35 mm. (Wallach (5) reports 98–100°/55 mm., and Rupe and Klemm (6) report 85–87°/14 mm.). The semicarbazide was prepared, m. 188°, resolidifying and remelting at 224–8°; Wallach (5) records m. 183–5°, remelting at 220°.

*Methylation of 2-Formylcyclohexanone* (1). A three-neck flask fitted as before was used; 100 cc. of absolute methanol was placed in it. 1.65 g. (0.072 mole) of sodium was added and the solution cooled, 8.7 g. (0.072 mole) of 2-formylcyclohexanone was added slowly, a yellow color developing. A solution of 12 g. (0.085 mole) of methyl iodide in 10 cc. of absolute methanol was added slowly. The mixture stood overnight. It was slightly alkaline next day, but a 40 cc. sample was pipetted into a polarimeter tube and a reading taken. No optical activity was observed.

The 40 cc. was returned to the flask which was heated on the water bath for an hour. The solution was then neutral to litmus, so the methanol was removed under reduced pressure. The residue—a yellow oil—was treated with 15 cc. of water and extracted three times with 15 cc. of chloroform, which became yellow. This was dried over  $\text{CaCl}_2$ . Examination of the chloroform solution in the polarimeter revealed no optical activity.

*2-Formylcyclohexanone* (2). A second lot of 2-formylcyclohexanone was prepared by the method used in (1). 14.55 g. of sodium, 38 g. of methyl formate, and 62 g. of cyclohexanone (0.633 mole each) were used. 23.9 g. of 2-formylcyclohexanone was formed, b.94–6°/27–9 mm.

*Methylation of 2-Formylcyclohexanone* (2). The procedure used was identical with that used in (1), 23.8 g. of 2-formylcyclohexanone, 26.8 g. of methyl iodide, and 4.35 g. of sodium (0.189 mole in each case) were used. The same procedure as before was followed; the product did not rotate the plane of polarized light.

*Summary.* An attempt to prepare optically active 2-methyl-2-formylcyclohexanone from optically inactive 2-formylcyclohexanone was unsuccessful.

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# THE DECOMPOSITION OF AMMONIUM-DEUTERO CHLORIDE

BY SAMUEL B. KNIGHT AND EDWIN C. MARKHAM

*Contribution from the Venable Chemical Laboratory of the University of North Carolina*

It is to be expected that all four hydrogen atoms of the ammonium ion have the same degree of reactivity and that each one stands an equal chance of being removed when ammonia is formed by the decomposition of ammonium chloride with a base. While the fourth hydrogen atom (the one picked up from HCl) may be considered to be bound by a coordinate (or semi-polar) bond in a certain sense as far as origin is concerned, there is no reason to believe, once it is bound to the nitrogen atom, that the bond is any different from the three other bonds.

We have been interested in substituting a deuterium atom for one of the four hydrogens in ammonium chloride to determine whether or not its bond with the nitrogen is of the same strength as the ordinary hydrogen nitrogen bond. Accordingly, pure mono-deutero ammonium chloride was prepared by the reaction of ammonia with pure deuterium chloride. Decomposition of this compound by reaction with a base should yield a gas mixture containing 25% NH<sub>3</sub> and 75% NH<sub>2</sub>D, provided there is no difference in bond strength.

## EXPERIMENTAL

*Preparation of NH<sub>2</sub>DCl.* Heavy water, 99.6% pure D<sub>2</sub>O, was slowly vaporized through a tube filled with anhydrous magnesium chloride, which was heated to 600° (1). At this temperature, the composition of the resulting gas is about 75% DCl and 25% D<sub>2</sub>O. The D<sub>2</sub>O was absorbed in "desicchiora" while the DCl was frozen out with liquid air. This gaseous DCl was admitted cautiously into a reaction chamber which contained ammonia previously dried by passage over calcium oxide. In this chamber the gases reacted to form NH<sub>2</sub>DCl. Finally, the system was evacuated and the solid NH<sub>2</sub>DCl sublimed into a heated chamber containing dry calcium oxide in which NH<sub>3</sub> and NH<sub>2</sub>D were generated. After passing through a calcium oxide drying tube, the gases were collected in a weighing bulb of 106.00 cc. volume. By accurately weighing the bulb and contents, the density of the gas mixture and hence the apparent molecular weight and percentage composition were determined.

*Weighing the Gas.* A microbalance sensitive to 0.005 mg. was mounted in a manner similar to that employed by Steele and Grant (2). It was found that the full sensitivity of the balance could be utilized and at the same time the balance was ideal for the rapid weighing of a known volume of gas.

The balance was housed in an air-tight brass case with a glass window on one side. The oscillation of the balance and position of the zero point were observed by means of a spot of light reflected from a small mirror on the beam through the glass window in the case onto a scale a few feet away. The balance was mounted

on a table resting on a concrete pillar driven into the ground. By varying the air pressure within the case, the buoyancy of the bulb was changed and by measuring the pressure change on a manometer by means of a cathetometer, the weight applied could be calculated.

The Pyrex glass weighing bulb was fitted with a stopcock which, though small, was so perfectly ground that when properly greased no difficulty was experienced with leaking. When being weighed, this bulb was attached to the right arm of the balance with a platinum wire and counterpoised with a plumb-bob of Pyrex glass.

For reasons of experimental convenience the balance was calibrated by weighing the glass bulb filled with clean dry air at 0° and a known pressure. The bulb was transferred to the balance and allowed to come to thermal equilibrium with the case. Knowing the pressure of the air in the bulb and the volume of the

TABLE I  
*Molecular Weight Values of Ammonia Formed by Decomposition of  
Ammonium-deutero Chloride*

TRIAL NUMBER	PRESSURE OF GAS IN BULB	WEIGHT OF GAS FROM GRAPH	OBSERVED MOLECULAR WEIGHT	LESS OBSERVED MOLECULAR WEIGHT OF ORDINARY NH <sub>3</sub>
1	753.63	0.08472	18.06	0.75
2	706.83	0.07892	18.00	0.69
3	707.90	0.07947	18.04	0.73
4	700.15	0.07855	18.04	0.73
5	711.33	0.07970	18.00	0.69
6	708.65	0.07922	18.02	0.71
7	711.60	0.07963	18.00	0.69
8	726.75	0.08154	18.03	0.72
9	710.30	0.07961	18.01	0.70

latter, 106.00 cc., the weight of air could be calculated from its known density at 0° and 760 mm. by means of the ideal gas law. The density value was obtained from the International Critical Tables. A calibration curve was constructed by plotting balance-case pressures against known weights of air in the bulb. The above procedure was repeated a limited number of times with pure ammonia instead of air in order to obtain relative weights from which the apparent molecular weight of ammonia could be calculated. The calculation of the molecular weight of ammonia from the relative weights of air and ammonia does not give the absolute value of the molecular weight of the latter because neither gas obeys the ideal gas law. Thus the apparent molecular weight of ammonia as determined by this method was 17.31 ( $\pm 0.02$ ) instead of 17.03. The value of 17.31 served our purpose as well as the true molecular weight because the decomposition products, ammonia and deutero-substituted ammonia, were weighed in the same manner and the apparent molecular weight compared with that of pure ammonia. Such a comparison of the apparent molecular weights indicates exactly the *differences* between the molecular weights and therefore the dif-

ferences in composition of the gases. Whereas, neither pure ammonia nor deutero-ammonia obeys the ideal gas law both are expected to deviate by the same amount in view of the fact that the physical properties of the two are almost identical.

During the course of the investigation, the constancy of the weight of the bulb was checked by filling it with ammonia at some known pressure and comparing it with the calibration curve. The zero point of the balance was determined before and after each run, by means of a sealed off glass bulb which weighed almost the same ( $\pm 0.1$  mg.) as the bulb used in the molecular weight determination.

*Results of Decomposition of  $\text{NH}_3\text{DCl}$ .* The fourth column in Table I gives the observed molecular weight of the gas resulting from the decomposition of  $\text{NH}_3\text{DCl}$ . The last column gives the difference after the observed average molecular weight of ordinary ammonia has been subtracted.

The average molecular weight of the gas mixture is 0.71 higher than the average molecular weight obtained for ordinary ammonia.

TABLE II

*Molecular Weight Values of Ammonia Formed by Decomposition of 71% Ammonium Deutero Chloride and 29% Ammonium Chloride Mixtures*

TRIAL NUMBER	PRESSURE OF GAS IN BULB	WEIGHT OF GAS FROM GRAPH	OBSERVED MOLECULAR WEIGHT	LESS OBSERVED MOLECULAR WEIGHT OF ORDINARY $\text{NH}_3$
1	742.73	0.08216	17.78	0.47
2	725.75	0.08044	17.81	0.50
3	723.55	0.08010	17.79	0.48
			Mean 0.48	

## DISCUSSION OF RESULTS

The experimental results show that the difference between the molecular weight of the gas mixture ( $\text{NH}_3\text{D}$  and  $\text{NH}_3$ ) and that of pure ammonia is 0.71. If the gas were pure  $\text{NH}_3\text{D}$ , the molecular weight would be greater than that of pure ammonia by 1.00. Therefore, the composition of the gas mixture is 71%  $\text{NH}_3\text{D}$  and 29%  $\text{NH}_3$ . If deuterium and hydrogen were removed with equal ease from the ammonium ion, the gas would be 75%  $\text{NH}_3\text{D}$  and 25%  $\text{NH}_3$ . Thus, deuterium, in this case, reacts more readily, and the bond between nitrogen and deuterium must be weaker than that between nitrogen and hydrogen.

The fact that the nitrogen-deuterium bond is weaker than the nitrogen-hydrogen bond is to be explained as being due to the greater mass of the deuterium atom and not to a difference in the mode of formation of the bond.

As stated previously an ammonium salt containing one deuterium atom made from  $\text{NH}_3$  and  $\text{DCl}$  decomposes to give a gas mixture of apparent molecular weight 18.02. Similarly, an ammonium salt,  $\text{NH}_3\text{DHCl}$ , made from  $\text{NH}_3\text{D}$  and  $\text{HCl}$  should decompose in the same manner. To prove this point,  $\text{NH}_3\text{DHCl}$  was prepared not in the pure state but in a 71-29 ratio with  $\text{NH}_3\text{Cl}$  by the action of  $\text{HCl}$  on the original gas mixture of 71%  $\text{NH}_3\text{D}$  and 29%  $\text{NH}_3$ . Decomposi-

tion of this  $\text{NH}_2\text{D}\text{HCl}-\text{NH}_4\text{Cl}$  salt mixture should yield a gas having an apparent molecular weight of 17.81. The results in Table II show an average apparent molecular weight of 17.79 ( $\pm 0.02$ ).

Whereas, it is theoretically possible for a hydrogen-deuterium exchange to have taken place in the gas phase between  $\text{NH}_3$  and  $\text{NH}_2\text{D}$  on the one hand and  $\text{HCl}$  and  $\text{DCl}$  on the other hand, it is not likely that such is the case. The data in Table II support the point of view that no appreciable exchange takes place in the gas phase during the time of the decomposition of the salt and the subsequent absorption of the  $\text{HCl}$ - $\text{DCl}$  gases.

#### SUMMARY

1. Ammonium-deutero chloride has been prepared by the interaction of pure deuterium chloride and ammonia.
2. Decomposition of the ammonium-deutero chloride with a base yields a gas mixture of 71%  $\text{NH}_2\text{D}$  and 29%  $\text{NH}_3$ . Assuming deuterium and hydrogen to have the same reactivity, a gas mixture of 75%  $\text{NH}_2\text{D}$  and 25%  $\text{NH}_3$  is expected.
3. The weakness of the nitrogen-deuterium bond as compared with the nitrogen-hydrogen bond is due to the greater mass of the deuterium atom.
4. Ammonium-deutero chloride was prepared by the interaction of  $\text{NH}_2\text{D}$  and  $\text{HCl}$ . This salt decomposed to give 71%  $\text{NH}_2\text{D}$  and 29%  $\text{NH}_3$ .

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# CONCERNING A CERTAIN RING OF HOMOGRAPHIES

By E. T. BROWNE

*Department of Mathematics, University of North Carolina*

1. INTRODUCTION. Let  $\rho$  and  $a_{ij}$  ( $i, j = 1, \dots, n$ ) be constants, real or complex, and consider the linear homogeneous transformation

$$(1) \quad \rho y_i = \sum_{j=1}^n a_{ij} x_j,$$

by which the set of variables  $(x_1, \dots, x_n)$  is transformed into the set  $(y_1, \dots, y_n)$ . If we denote by  $A$  the  $n$ -square matrix of the coefficients and by  $X$  and  $Y$ , respectively, one column matrices as follows:

$$A = \begin{pmatrix} a_{11} & \dots & a_{1n} \\ \dots & \dots & \dots \\ a_{n1} & \dots & a_{nn} \end{pmatrix}, \quad X = \begin{pmatrix} x_1 \\ \vdots \\ x_n \end{pmatrix}, \quad Y = \begin{pmatrix} y_1 \\ \vdots \\ y_n \end{pmatrix},$$

we can write the transformation (1) more compactly in the form

$$(2) \quad \rho Y = AX.$$

Here  $A$  is called the *matrix of the transformation*, and in case the determinant of  $A$  is different from zero, the transformation is said to be *non-singular*. The sets  $X$  and  $Y$  can be considered as components of vectors in an affine  $n$ -space, or as projective coordinates of points in  $(n - 1)$ -space. In the latter case we speak of (1) or (2) as a projective transformation, a *collineation* or a *homography*.

In the particular case in which  $A$  has the property

$$A^2 = kI \quad (k \neq 0)$$

where  $I$  is the  $n$ -square unit matrix, the transformation is said to be of *period two* and is called an *involution*. Since (2) can be divided through by  $\sqrt{k}$  and  $\rho/\sqrt{k}$  can be written as a new constant  $\sigma$ , every involution can be written in the form

$$\sigma Y = AX,$$

where

$$(3) \quad A^2 = I.$$

In an earlier paper\* the author considered pairs of  $n$ -square matrices  $A, B$  such that not only  $A^2 = I, B^2 = I$ , but such that in addition, if  $\lambda$  and  $\mu$  are any numbers, real or complex, such that  $\lambda + \mu \neq 0$ , then

$$(4) \quad D = \frac{\lambda A + \mu B}{\lambda + \mu} = (1 - \theta)A + \theta B \left( \theta = \frac{\mu}{\lambda + \mu} \right)$$

\* *Involutions that Belong to a Linear Class*, Annals of Mathematics, Second Series Vol. 29 (1928), pp. 483-492.

also possesses the property  $D^2 = I$ . Such a pair of matrices  $A, B$  are said to determine a linear class of involutions to which  $D$  belongs. Before proceeding further with the discussion, it will be convenient to enunciate without proof certain results of the earlier paper, a knowledge of which will be necessary for what follows.

- I. If  $A^2 = I$ ,  $A$  has no characteristic roots other than  $\pm 1$ .
- II. If  $A$  has a characteristic root of multiplicity  $m$  at  $+1$ , and therefore a characteristic root of multiplicity  $n - m$  at  $-1$ , the matrices  $A - I$  and  $A + I$  are of ranks  $n - m$  and  $m$ , respectively. Hence if  $A \neq \pm I$ ,  $m$  satisfies the inequalities  $0 < m < n$ .
- III. There exists a non-singular matrix  $P$  such that  $P^{-1}AP$  assumes the canonical form

$$(5) \quad P^{-1}AP = \begin{pmatrix} I_m & 0 \\ 0 & -I_{n-m} \end{pmatrix},$$

where  $I_m$  is an  $m$ -square unit matrix containing  $+1$ 's in the diagonal and zeros elsewhere.

IV. Corresponding to the characteristic root  $+1$ , there is an  $m$ -dimensional linear vector space  $\mathcal{G}_1$  of fixed points  $X$  such that  $AX = X$ , i.e.,  $(A - I)X = 0$ ; and corresponding to the root  $-1$ , there is an  $(n - m)$ -dimensional space  $\mathcal{G}_{-1}$  of fixed points  $Y$  such that  $(A + I)Y = 0$ , and the two spaces  $\mathcal{G}_1, \mathcal{G}_{-1}$  have no vector in common except the zero vector.

V. A necessary condition that  $A$  and  $B$  determine a linear class is that  $A$  and  $B$  be similar; i.e., if  $A$  has a characteristic root of multiplicity  $m$  at  $+1$ , so also has  $B$ .

VI. If the condition laid down in V is satisfied, a sufficient condition that  $A$  and  $B$  determine a linear class is that either the spaces  $\mathcal{G}_1, \mathcal{B}_1$  coincide or the spaces  $\mathcal{G}_{-1}, \mathcal{B}_{-1}$  coincide. Since in the latter case  $-A$  and  $-B$  determine a linear class in which  $\mathcal{G}_1 = \mathcal{B}_1$ , we can without loss of generality, and shall, suppose that the former condition holds.

In this paper we suppose that  $n$  is even ( $n = 2m$ ) so that each of the spaces  $\mathcal{G}_1, \mathcal{G}_{-1}, \mathcal{B}_1, \mathcal{B}_{-1}$  is of dimension  $m$ . Furthermore, as will appear in what follows, it will be convenient to change the sign of  $B$  so that  $\mathcal{G}_1 = \mathcal{B}_{-1}$  while  $\mathcal{G}_{-1}$  and  $\mathcal{B}_1$  have no vector in common. If then  $D = (1 - \theta)A - \theta B$ , it is easy to show that  $\mathcal{D}_1$  coincides with  $\mathcal{G}_1 = \mathcal{B}_{-1}$ , while as  $\theta$  changes  $\mathcal{D}_{-1}$  sweeps out a hypersurface  $\Sigma$  of which  $\mathcal{D}_{-1}$  is a generator.

The conditions that  $\mathcal{G}_1 = \mathcal{B}_{-1}$  are obtained from results found in the earlier paper by changing the sign of  $B$  and are merely

$$(6) \quad (A - I)(B - I) = (B + I)(A + I) = 0.$$

We now show that we can find a third involution  $C$ , similar to  $A$  and  $B$ , and uniquely determined by the latter, such that

$$(7) \quad \mathcal{B}_1 = \mathcal{C}_{-1}, \quad \mathcal{C}_1 = \mathcal{G}_{-1}.$$

Such a triple of involutions we shall call a *symmetrical triple*. These three involutions have the property not only that

$$A^2 = B^2 = C^2 = I,$$

but if

$$(8) \quad \begin{aligned} D &= (1 - \theta)A - \theta B, \\ E &= (1 - \phi)B - \phi C, \\ F &= (1 - \tau)C - \tau A, \end{aligned}$$

then also  $D^2 = E^2 = F^2 = I$  for every  $\theta, \phi$  and  $\tau$ . If in (6) we permute  $A, B$  and  $C$  cyclically, we obtain as the conditions that (7) be satisfied

$$(9) \quad (B - I)(C - I) = (C + I)(B + I) = 0,$$

$$(10) \quad (C - I)(A - I) = (A + I)(C + I) = 0.$$

Under these conditions it is easy to verify that if  $x, y$  and  $z$  are any scalars, then

$$(11) \quad H = xA + yB + zC$$

has the property

$$(12) \quad H^2 = k^2 I,$$

where

$$(13) \quad k^2 = f(x, y, z) = x^2 + y^2 + z^2 - 2xy - 2xz - 2yz.$$

Thus the triple  $A, B, C$  can be looked upon as a *basis* for a two parameter family of involutions. Since in the projective geometry of  $n - 1$  dimensions, the involutions  $H$  and  $\frac{1}{k}H$  ( $k \neq 0$ ) have essentially the same properties, we have thus a 1-1 correspondence between the *involutions*  $H$  in (11) and *points*  $(x, y, z)$  in the plane. To a point  $(x, y, z)$  on the conic

$$(14) \quad S: \quad f(x, y, z) = 0$$

for which  $k = 0$ , there corresponds no *proper* involution  $H$ , since then  $H^2 = 0$ . We shall, however, call such an involution a *parabolic*\* involution of the family.

**2. CANONICAL FORM OF A SYMMETRICAL TRIPLE OF INVOLUTIONS. THEOREM I.** *If  $A, B$ , and  $C$  constitute a symmetrical triple of involutions and if  $P$  is any non-singular matrix, then  $U = P^{-1}AP$ ,  $V = P^{-1}BP$ ,  $W = P^{-1}CP$  also constitute a symmetrical triple.*

For from (6) we have

$$(U - I)(V - I) = P^{-1}(A - I)PP^{-1}(B - I)P = P^{-1}(A - I)(B - I)P = 0.$$

\* Winger, *An Introduction to Projective Geometry*, Boston, 1923, pp. 160-162; also Ex. 2, p. 168.

Similarly, the second condition in (6) and (9), (10) hold with  $A, B, C$ , replaced by  $U, V, W$ .

Now by III, section 1,  $P$  can always be so chosen that  $U$  is of the form

$$U = \begin{pmatrix} I_m & 0 \\ 0 & -I_m \end{pmatrix}.$$

If then we partition  $V$  similarly

$$V = \begin{pmatrix} V_1 & V_2 \\ V_3 & V_4 \end{pmatrix} \quad .$$

where each of the  $V_i$  is an  $m$ -square matrix, and impose the conditions (6), it is easy to verify that  $V_1 = -I_m$ ,  $V_3 = 0$ ,  $V_4 = I_m$  while  $V_2$  is arbitrary. That is

$$V = \begin{pmatrix} -I_m & V_2 \\ 0 & I_m \end{pmatrix}.$$

Similarly, from (10) it is easy to verify that  $W$  is of the form

$$W = \begin{pmatrix} -I_m & 0 \\ W_3 & I_m \end{pmatrix}.$$

The conditions (9) then lead to the relation  $V_2 W_3 = -4I_m$ , so that neither  $V_2$  nor  $W_3$  is singular. If we take

$$P = \begin{pmatrix} R & 0 \\ 0 & S \end{pmatrix}$$

where  $R$  and  $S$  are non-singular, under the transformation  $P^{-1}UP$ ,  $U$  is unchanged while  $V$  is transformed into a matrix of the same type with  $V_2$  replaced by  $R^{-1}V_2S$ . Since  $V_2$  is non-singular, we can choose  $R$  and  $S$  so that  $V_2 = 2I_m$ . Hence  $W_3 = -2I_m$ . We therefore have as canonical forms for a symmetrical triple of involutions the three matrices:

$$(15) \quad A = \begin{pmatrix} 1 & 0 \\ 0 & -1 \end{pmatrix} \quad B = \begin{pmatrix} -1 & 2 \\ 0 & 1 \end{pmatrix} \quad C = \begin{pmatrix} -1 & 0 \\ -2 & 1 \end{pmatrix}.$$

Here we have used the symbol 1 to stand for the unit matrix  $I_m$ .

3. THE ALGEBRA  $R(A, B, C, I)$ . THEOREM II. *The matrices  $A, B, C, I$  are linearly independent.*

This theorem follows directly from use of the canonical forms in (15). Otherwise it can be proved directly as follows. Suppose that

$$xA + yB + zC + wI = 0.$$

On multiplying through on the right and on the left by  $A$  and adding, we have, on making use of the relations (6), (9) and (10),

$$(2x - 2y - 2z)I + 2wA = 0.$$

Hence, since  $A \neq kI$ ,  $w = 0$ ,  $x = y + z$ . Similarly, using the multipliers  $B$  and  $C$ , we obtain  $y = x + z$ ,  $z = x + y$ . Hence,  $x = y = z = w = 0$ .

**THEOREM III.** *The matrices  $A, B, C, I$  form the basis of an algebra\*  $R(A, B, C, I)$  of order four over the field of complex numbers.*

In fact, by (6), (9) and (10) the multiplication table of the algebra is as follows:

	$A$	$B$	$C$	$I$
$A$	$I$	$A + B - I$	$-C - A - I$	$A$
$B$	$-A - B - I$	$I$	$B + C - I$	$B$
$C$	$C + A - I$	$-B - C - I$	$I$	$C$
$I$	$A$	$B$	$C$	$I$

Obviously, in view of the relations (6), (9) and (10),  $R$  is not a *division algebra*. Subalgebras of this algebra are easily seen to be those with bases:  $(A, I)$ ,  $(B, I)$ ,  $(C, I)$ ,  $(A, B, I)$ ,  $(A, C, I)$ ,  $(B, C, I)$ .

Consider now an arbitrary element  $D$  of the algebra

$$(17) \quad D = xA + yB + zC + wI = H + wI,$$

where  $H$  is given by (11). If we put  $\bar{D} = H - wI$ , we have

$$D\bar{D} = \bar{D}D = H^2 - w^2I = (k^2 - w^2)I.$$

If  $w^2 \neq k^2$ , i.e., if  $|D| \neq 0$ , then

$$D^{-1} = \frac{\bar{D}}{k^2 - w^2}.$$

We therefore have the theorem:

**THEOREM IV.** *Any element  $D = xA + yB + zC + wI$  of the algebra  $R(A, B, C, I)$ , for which  $k^2 - w^2 = x^2 + y^2 + z^2 - 2xy - 2xz - 2yz - w^2 \neq 0$ , has an inverse*

$$D^{-1} = \frac{1}{k^2 - w^2} \bar{D}$$

where

$$(18) \quad \bar{D} = xA + yB + zC - wI.$$

From (17) we have

$$D^2 = (k^2 + w^2)I + 2wH,$$

whence, on replacing  $H$  by  $D - wI$ , we obtain

$$(19) \quad D^2 - 2wD + (w^2 - k^2)I = 0.$$

**THEOREM V.** *Every element (17) of the algebra  $R(A, B, C, I)$  satisfies a quadratic equation (19).  $D$  will be an involution if, and only if,  $w = 0$ .*

**4. FIXED ELEMENTS UNDER A TRANSFORMATION.** Let us now investigate the fixed elements under the transformations of the ring. It will be convenient to consider  $H$  first.

\* Dickson, *Algebras and their Arithmetics*, University of Chicago Press, 1923, pp. 9-24; cf. also MacDuffee, *An Introduction to Abstract Algebra*, New York, 1940, pp. 277-278. We shall frequently refer to this algebra as the *ring  $R(A, B, C, I)$* .

For  $k \neq 0$   $H$  has characteristic roots of multiplicity  $m = n/2$  at  $+k$  and at  $-k$ . Corresponding to these roots there are linear spaces  $\mathcal{K}_1$  and  $\mathcal{K}_{-1}$  of fixed points. If  $Z$  is a point not in one of these spaces, and if, under  $H$ ,  $Z$  is transformed into the point  $W$ , on the line  $ZW$  there is one point  $X$  of  $\mathcal{K}_1$  and one point  $Y$  of  $\mathcal{K}_{-1}$ , and the pair  $Z, W$  separates the pair  $X, Y$  harmonically. For  $n = 2$ ,  $H$  is the familiar involution of points on a point row with  $\mathcal{K}_1$  and  $\mathcal{K}_{-1}$  as double points. For  $n = 4$ ,  $H$  is a line reflection whose axes are the lines  $\mathcal{K}_1$  and  $\mathcal{K}_{-1}$ .

If  $k = 0$ ,  $H$  is a parabolic involution. There is no proper space of fixed points of  $H$ , but there is a linear space  $\mathcal{K}_0$  of points  $X$  such that  $HX = 0$ . We call this the *null space* of  $H$ . If  $Z$  is any point not in  $\mathcal{K}_0$ , under  $H$ ,  $Z$  is transformed into a point  $W$  of  $\mathcal{K}_0$  such that every point of the line  $ZW$  is transformed into this same point  $W$ .

Next consider the transformation  $D = H + wI$  ( $w \neq 0$ ).

If  $k \neq 0$ ,  $D$  has the characteristic roots  $w \pm k$ , each of multiplicity  $m$  corresponding to which there are two spaces, precisely the spaces  $\mathcal{K}_1$  and  $\mathcal{K}_{-1}$  of  $H$ , which are spaces of fixed points (or one of which is a null space in case  $w = \pm k$ ), of  $D$ . If  $Z$  is a point not in one of these spaces, then, under  $D$ ,  $Z$  is transformed into a point  $W$  such that on the line  $ZW$ , there is one point  $X$  of  $\mathcal{K}_1$  and one point  $Y$  of  $\mathcal{K}_{-1}$  and the double ratio of the points  $X, Y, Z, W$  is  $\frac{w+k}{w-k}$ .  $D$  is the familiar *biaxial homography*.

If  $k = 0$ ,  $D$  has a characteristic root of multiplicity  $n$  at  $w$ , corresponding to which there is a space of fixed points of  $D$ . This space is precisely the null space  $\mathcal{K}_0$  of  $H$ . If  $Z$  is any point not in  $\mathcal{K}_0$  and if  $W$  is the point in  $\mathcal{K}_0$  to which  $Z$  is transformed by  $H$ , under  $D$ ,  $Z$  is transformed into a point on the line  $ZW$ .

## 5. FORMATION OF OTHER SYMMETRICAL TRIPLES.

Let  $A, B$  and  $C$  be a symmetrical triple of involutions satisfying the conditions (6), (9) and (10) so that

$$\mathcal{A}_1 = \mathcal{B}_{-1}, \quad \mathcal{B}_1 = \mathcal{C}_{-1}, \quad \mathcal{C}_1 = \mathcal{A}_{-1}.$$

If we write  $K = (1 - \theta)A - \theta B$ , it is known that, for every  $\theta$ ,  $\mathcal{K}_1$  coincides with  $\mathcal{A}_1 = \mathcal{B}_{-1}$ , while as  $\theta$  changes  $\mathcal{K}_{-1}$  sweeps out a hypersurface  $\Sigma$  and by proper choice of  $\theta$  may be chosen as any of the generating spaces of  $\Sigma$  except  $\mathcal{K}_1$  itself. Similarly, if we form

$$T = (1 - \phi)B - \phi C$$

then for every choice of  $\phi$

$$\mathcal{T}_1 = \mathcal{B}_1 = \mathcal{C}_{-1}.$$

If now we put  $L = -T$  so that

$$L = (\varphi - 1)B + \phi C$$

then

$$\mathcal{L}_{-1} = \mathcal{B}_1 = \mathcal{C}_{-1}.$$

The question now arises as to whether it is possible so to choose  $\theta$  and  $\phi$  that

$$\mathcal{L}_1 = \mathcal{K}_{-1},$$

in which case the involutions  $L, K, B$  in that order form another symmetrical triple. The conditions for this are (6) with  $A$  and  $B$  replaced by  $L$  and  $K$ , respectively; i.e.,

$$(20) \quad (L - I)(K - I) = (K + I)(L + I) = 0.$$

or

$$[(\phi - 1)B + \phi C - I][(1 - \theta)A - \theta B - I] = 0$$

$$[(1 - \theta)A - \theta B + I][(\phi - 1)B + \phi C + I] = 0.$$

On multiplying out and making use of the multiplication table (16), we find that these conditions are satisfied if, and only if,  $\theta$  and  $\phi$  are connected by the relation

$$(21) \quad \theta\phi - \phi + 1 = 0; \quad \text{i.e., } \theta = \frac{\phi - 1}{\phi}.$$

We therefore have the Theorem:

**THEOREM VI.** *If  $A, B$ , and  $C$  are a symmetrical triple of involutions so that  $\mathcal{A}_1 = \mathcal{B}_{-1}$ ,  $\mathcal{B}_1 = \mathcal{C}_{-1}$ ,  $\mathcal{C}_1 = \mathcal{A}_{-1}$ , and if we form*

$$L = (\phi - 1)B + \phi C$$

$$K = \frac{1}{\phi} A + \frac{1 - \phi}{\phi} B,$$

*then the involutions  $L, K$  and  $B$  in that order form a new symmetrical triple in which  $\mathcal{K}_1 = \mathcal{B}_{-1}$ ,  $\mathcal{B}_1 = \mathcal{L}_{-1}$  are fixed spaces belonging to the original triple, while  $\mathcal{L}_1 = \mathcal{K}_{-1}$  can by proper choice of  $\phi$  be chosen to be any one of the generating spaces of the hypersurface  $\Sigma$ .*

By forming combinations of  $L, K$  and  $B$  similar to those by which the latter were formed from  $A, B$  and  $C$ , we may build up other symmetrical triples. Thus

$$M = (\alpha - 1)K + \alpha B = \frac{1}{\phi} [(\alpha - 1)A + (\alpha + \phi - 1)B]$$

$$N = \frac{1}{\alpha} L + \frac{1 - \alpha}{\alpha} K = \frac{1}{\alpha\phi} [(1 - \alpha)A + (\phi - 1)(\alpha + \phi - 1)B + \phi^2 C]$$

$$K = \frac{1}{\phi} [A + (1 - \phi)B]$$

form a symmetrical triple in which now  $\mathcal{N}_1 = \mathcal{K}_{-1}$  is arbitrary,  $\mathcal{K}_1 = \mathcal{M}_{-1}$  is the fixed space  $\mathcal{B}_{-1}$  of the original triple while  $\mathcal{M}_1 = \mathcal{N}_{-1}$  can by proper choice of  $\alpha$  be taken to be any of the generating spaces of  $\Sigma$  except  $\mathcal{M}_{-1}$ . Hence,  $N$  is an involution whose fixed spaces can by proper choice of the parameters  $\alpha$  and  $\phi$  be chosen as any two of the generating spaces of  $\Sigma$ . In other words,  $N$  is the general involution of the family with basis  $A, B, C$ .

Continuing in this manner we build up

$$U = (\lambda - 1)N + \lambda K$$

$$= \frac{1}{\alpha\phi} [(\alpha + \lambda - 1)A + (\phi - 1)(\lambda\phi - \alpha - \lambda - \phi + 1)B + \phi^2(\lambda - 1)C],$$

$$V = \frac{1}{\lambda} M + \frac{1 - \lambda}{\lambda} N = \frac{1}{\alpha\lambda\phi} [(\alpha - 1)(\alpha + \lambda - 1)A - (\alpha + \phi - 1)(\lambda\phi - \alpha - \lambda - \phi + 1)B + \phi^2(1 - \lambda)C],$$

$$W = N = \frac{1}{\alpha\phi} [(1 - \alpha)A + (\phi - 1)(\alpha + \phi - 1)B + \phi^2 C];$$

a symmetrical triple whose three spaces  $\mathfrak{U}_1 = \mathfrak{V}_{-1}$ ,  $\mathfrak{V}_1 = \mathfrak{W}_{-1}$ ,  $\mathfrak{W}_1 = \mathfrak{U}_{-1}$  are any three of the generating spaces of  $\Sigma$ . Indeed, this triple is the most general symmetrical triple built up on the triple  $A, B, C$ .

6. THE GENERAL INVOLUTION OF THE RING  $R(A, B, C, I)$ . It has been shown that if  $A, B$  and  $C$  form a symmetrical triple and if  $H = xA + yB + zC$ , where  $x, y$  and  $z$  are any numbers such that  $k^2 = f(x, y, z)$  in (13) is different from zero, then  $\frac{1}{k}H$  is an involution of the ring  $R$ . Since  $H$  is expressed homogeneously in  $x, y$  and  $z$ , we may suppose that this latter set is divided through by the proper factor so that  $k^2 = 1$ . Since by the preceding section it was shown that  $N$  above is an expression for the general involution of the ring, it should be possible by proper choice of  $\alpha$  and  $\phi$  to make  $N$  identical with  $H$ . The conditions for this are, in addition to  $k^2 = 1$ ,

$$\frac{1 - \alpha}{\alpha\phi} = x, \quad \frac{(1 - \phi)(1 - \alpha - \phi)}{\alpha\phi} = y, \quad \frac{\phi}{\alpha} = z.$$

From the first and the third of these equations, we have on eliminating  $\phi$ ,

$$xz\alpha^2 + \alpha - 1 = 0,$$

whence

$$\alpha = \frac{-1 \pm \sqrt{1 + 4xz}}{2xz} = \frac{-1 \pm (x - y + z)}{2xz};$$

and therefore

$$\phi = \frac{-1 \pm (x - y + z)}{2x}.$$

Assuming that none of  $x, y, z$  is zero, it follows from  $k^2 = 1$  that  $x - y + z \neq \pm 1$ . If therefore we take the upper sign and multiply numerator and denominator of  $\alpha$  through by  $x - y + z + 1$ ,  $\alpha$  and  $\phi$  can be written in the form

$$\alpha = \frac{2^{\frac{1}{2}}}{x - y + z + 1}, \quad \phi = \frac{2z}{x - y + z + 1}.$$

With these values of  $\alpha$  and  $\phi$  the coefficient of  $B$  in  $N$  is easily seen to reduce to  $y$  so that  $N$  is identical with  $H$ .

### 7. HARMONIC PROPERTIES.\* We define the two involutions

$$H = xA + yB + zC,$$

$$H' = x'A + y'B + z'C,$$

of the ring  $R$  to be *harmonic* provided the corresponding points  $(x, y, z)$  and  $(x', y', z')$  in the plane are *conjugate* with respect to the conic  $S$  in (14); i.e., provided

$$(24) \quad x' \frac{\partial f}{\partial x} + y' \frac{\partial f}{\partial y} + z' \frac{\partial f}{\partial z} = 0.$$

Clearly the harmonic relation as thus defined is a reciprocal one as it should be. The *parabolic* involutions of the ring are *self harmonic*.

In particular, the conditions that  $H$  be harmonic to  $A$ ,  $B$  and  $C$  are

$$\frac{\partial f}{\partial x} = 2(x - y - z) = 0, \quad \frac{\partial f}{\partial y} = 2(-x + y - z) = 0,$$

$$\frac{\partial f}{\partial z} = 2(-x - y + z) = 0,$$

respectively.

Let us now suppose that the three involutions  $V$ ,  $W$ , and  $U$  given by (23) in that order are harmonic to  $A$ ,  $B$  and  $C$ , respectively. The conditions for this are easily verified to be

$$(25) \quad \begin{aligned} \phi(\alpha - 2) &= 0, \\ 2\phi(\alpha + \lambda + \phi - \lambda\phi - 1) &= \alpha\lambda\phi, \\ 2(\alpha + \lambda + \phi - \lambda\phi - 1) &= \alpha\phi. \end{aligned}$$

Since  $\phi \neq 0$ ,  $\alpha = 2$ ; whence from the last two equations  $\lambda = \phi$ , where  $\phi$  is a root of the equation

$$(26) \quad \phi^2 - \phi - 1 = 0.$$

If we denote by  $\phi_1$  and  $\phi_2$  the roots of this quadratic and substitute these values into (23), we obtain

$$(27) \quad \begin{aligned} V &= \frac{1}{2}(A + \phi_1 B + \phi_2 C) \\ U &= \frac{1}{2}(\phi_2 A + B + \phi_1 C) \\ W &= \frac{1}{2}(\phi_1 A + \phi_2 B + C). \end{aligned}$$

The matrix of the coefficients in (27) is *orthogonal*, as we might expect, since the harmonic relation is a reciprocal one. Had we imposed the conditions that

\* This definition is justified by the easily established fact that if  $n=2$ , so that  $H$  and  $H'$  are involutions of points on a point row, the double points of  $H'$  will separate harmonically the double points of  $H$ .

$U, V, W$ , in that order, or  $W, U, V$  in that order separate  $A, B, C$ , harmonically; we should have arrived at the same expressions for  $U, V, W$ , (or  $W, U, V$ ) but in these cases the computation is not so simple.

We may therefore state the theorem:

**THEOREM VII.** *If  $A, B$ , and  $C$  are a symmetrical triple of involutions of the ring  $R(A, B, C, I)$  there are exactly two symmetrical triples  $V, U, W$  of the ring which are harmonic to  $A, B, C$ , respectively, and their expressions are given by (27).*

### 8. AUTOMORPHISMS OF THE RING.

We now prove the theorem:

**THEOREM VIII.** *If  $D = aA + bB + cC$  is any involution of the ring  $R(A, B, C, I)$  so that  $D^2 = I$ , i.e.,*

$$(28) \quad (a - b - c)^2 - 4bc = 1,$$

*there exists a non-singular element of the ring  $P = xA + yB + zC + wI$  such that  $P^{-1}AP = D$ .*

This theorem is proved most readily if we take  $A, B$  and  $C$  in the canonical forms (15). We then have as expressions for  $P$  and  $D$ ,

$$P = \begin{pmatrix} x - y - z + w & 2y \\ -2z & -x + y + z + w \end{pmatrix}; D = \begin{pmatrix} a - b - c & 2b \\ -2c & -a + b + c \end{pmatrix}.$$

We are to show that we can find four numbers  $x, y, z, w$  such that

$$(29) \quad |P| = w^2 - (x - y - z)^2 + 4yz \neq 0,$$

and such that  $AP = PD$ .

On forming the products of the matrices and equating corresponding elements we are led to the four homogeneous linear equations

$$\begin{aligned} (x - y - z + w)(a - b - c - 1) - 4cy &= 0 \\ b(x - y - z + w) + y(-a + b + c - 1) &= 0 \\ c(-x + y + z + w) + z(a - b - c + 1) &= 0 \\ (-x + y + z + w)(-a + b + c + 1) - 4bz &= 0. \end{aligned}$$

In view of the relation (28) the matrix of this set of equations is found to be of rank 2, so that we should expect it to be always possible to find sets  $x, y, z, w$  satisfying not only the conditions  $AP = PD$ , but also the condition that  $|P|$  in (29) shall be different from zero.

In fact, if  $a - b - c + 1 \neq 0$  so that  $bc \neq 0$ , we can take

$$(x, y, z, w) = (a + 1, b, c, 0)$$

as a set satisfying (30) and such that  $|P| = 4bc - (a - b - c + 1)^2 = -2(a - b - c + 1) \neq 0$ . If, however,  $a - b - c + 1 = 0$ , so that by (28),  $bc = 0$ , we take

$$\begin{aligned} \text{if } b = 0, c \neq 0, \quad (x, y, z, w) &= (c, -1, 1, c), & \text{where } & |P| = -4; \\ \text{if } c = 0, b \neq 0, \quad (x, y, z, w) &= (1, b, 1, b), & \text{where } & |P| = 4b. \end{aligned}$$

In case  $b = c = 0$ ,  $D$  reduces to  $A$ , and we can take  $P = A$ . Hence the theorem is proved.

We have also as a corollary the following theorem:

**THEOREM IX.** *Every automorphism of the ring  $R(A, B, C, I)$  is an inner automorphism.\**

In theorem I it was shown that if  $A, B$  and  $C$  form a symmetrical triple of involutions and if  $P$  is any non-singular matrix, then also  $U = P^{-1}AP$ ,  $V = P^{-1}BP$  and  $W = P^{-1}CP$  form a symmetrical triple. For a general choice of  $P$  these last three do not belong to the ring  $R(A, B, C, I)$ . If, however, we take

$$P = xA + yB + zC + wI, \quad |P| = w^2 - k^2 = 1$$

then by theorem IV,

$$P^{-1} = -xA - yB - zC + wI,$$

the triple  $U, V, W$  will belong to the ring, and we can so choose  $P$  that  $U$  will be any involution whatever of  $R$ . If we take  $P$  in the form above and use the multiplication table (16), we find that

$$U = u_1A + u_2B + u_3C,$$

$$V = v_1A + v_2B + v_3C,$$

$$W = w_1A + w_2B + w_3C.$$

where

$$T = \begin{pmatrix} u_1 & u_2 & u_3 \\ v_1 & v_2 & v_3 \\ w_1 & w_2 & w_3 \end{pmatrix} = \begin{pmatrix} -x^2 + (y - z + w)^2 & -2y(x - y - z - w) & -2z(x - y - z + w) \\ 2x(x - y + z - w) & -y^2 + (x - z - w)^2 & 2z(x - y + z + w) \\ 2x(x + y - z + w) & 2y(x + y - z - w) & -z^2 + (x - y + w)^2 \end{pmatrix}$$

In particular, we can so choose  $x, y, z$  and  $w$  that the involutions  $U, V$  and  $W$  are harmonic to  $A, B$ , and  $C$ , respectively. The conditions for this are

$$u_1 = u_2 + u_3, \quad v_1 = v_2 + v_3, \quad w_1 = w_2 + w_3.$$

The first of these relations is equivalent to

$$x^2 + y^2 + z^2 - w^2 - 2xy - 2xz + 6yz = 0,$$

which in view of the fact that

$$(32) \quad |P| = w^2 - x^2 - y^2 - z^2 + 2xy + 2xz + 2yz = 1,$$

yields

$$8yz = 1.$$

\* MacDuffee, *An Introduction to Abstract Algebra*, New York, 1940, pp. 54-56.

Similarly, the second and the third of the above relations yield

$$8xy = 8xz = 1.$$

Hence,

$$x = y = z = \pm 1/2\sqrt{2},$$

so that from (32),  $w^2 = 5/8$ . On substituting these values into (31), the matrix  $T$  is seen to be precisely

$$T = \frac{1}{2} \begin{pmatrix} 1 & \phi_1 & \phi_2 \\ \phi_2 & 1 & \phi_1 \\ \phi_1 & \phi_2 & 1 \end{pmatrix}$$

where  $\phi_1 = \frac{1 \pm \sqrt{5}}{2}$ , and  $\phi_2 = \frac{1 \mp \sqrt{5}}{2}$  are the roots of the quadratic

$$(26) \quad \phi^2 - \phi - 1 = 0.$$

These results agree with those found in Theorem VII.

**9. APPLICATIONS TO GEOMETRY.** For  $n = 2m = 2$ , the triple  $A, B, C$  are matrices of involutions of points on a point row, the fixed spaces  $\mathcal{G}_1, \mathcal{G}_{-1}$  of  $A$  are the double points of  $A$ , and the hypersurface  $\Sigma$  is the point row itself.

For  $n = 2m = 4$ , the transformation (1) with matrix  $A$  is a *line reflection*\* or a *harmonic biaxial homography*,† whose axes are the invariant spaces of  $A$ . The hypersurface  $\Sigma$  associated with the triple is a ruled quadric surface of which the fixed spaces are lines of a regulus  $\Gamma$ . The equation of  $\Sigma$  can be found by eliminating  $\theta$  from the four (of which precisely two are linearly independent) linear homogeneous equations in  $x_1, x_2, x_3, x_4$  whose coefficients are the rows of the matrix  $D + I$ , where  $D = (1 - \theta)A - \theta B$  (8).

In the particular case where  $A, B$  and  $C$  are in the canonical form (15) the equations from which  $\theta$  is to be eliminated are

$$(33) \quad \begin{aligned} 2x_1 - 2\theta x_3 &= 0, \\ 2x_2 - 2\theta x_4 &= 0, \end{aligned}$$

so that the equation of  $\Sigma$  is

$$(34) \quad x_1 x_4 - x_2 x_3 = 0.$$

The typical element (17) of the ring

$$D = xA + yB + zC + wI$$

for which  $|D| \neq 0$  in (32) is a biaxial homography whose axes (the spaces  $\mathcal{D}_1$  and  $\mathcal{D}_2$  of  $D$ ) are lines of a regulus  $\Gamma$  and by suitable choice of the parameters can be taken as any two lines of the regulus.

\* Cf. in this connection Veblen and Young, *Projective Geometry*, Vol. II, New York, 1918, pp. 259-279.

† Godeaux, L., *Leçons de Géométrie Projective*, Paris, 1933, pp. 202-204.

Under the transformation

$$T: \quad x_1 = x'_1, \quad x_2 = x'_3, \quad x_3 = x'_2, \quad x_4 = x'_4,$$

the quadric  $\Sigma$  is transformed into itself in such a way that lines of each regulus are transformed into lines of the other. Hence if we form

$$A' = T^{-1}AT, \quad B' = T^{-1}BT, \quad C' = T^{-1}CT,$$

we obtain a second symmetrical triple which is associated with the regulus  $\Gamma'$  in precisely the same way in which the triple  $A, B, C$  is associated with  $\Gamma$ . The canonical forms of  $A', B'$ , and  $C'$  are

$$(35) \quad A' = \begin{pmatrix} -1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & -1 & 0 \\ 0 & 0 & 0 & 1 \end{pmatrix}, \quad B' = \begin{pmatrix} 1 & 2 & 0 & 0 \\ 0 & -1 & 0 & 0 \\ 0 & 0 & 1 & 2 \\ 0 & 0 & 0 & -1 \end{pmatrix},$$

$$C' = \begin{pmatrix} 1 & 0 & 0 & 0 \\ -2 & -1 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & -2 & -1 \end{pmatrix}.$$

Accordingly if the numbers  $x', y', z', w'$  are so chosen that

$$D' = x'A' + y'B' + z'C' + w'I$$

is non-singular,  $D'$  is a biaxial homography whose axes may by proper choice of the parameters be taken as any two lines of the regulus  $\Gamma'$ .

Under  $D$  the quadric  $\Sigma$  is transformed into itself in such a way that each generator of  $\Gamma$  is transformed into a generator of  $\Gamma'$  while each generator of  $\Gamma'$  is transformed into itself. A similar remark holds for  $D'$ : Accordingly  $\Sigma$  is unaltered under the product  $G = DD' = D'D$  of the two transformations (which can easily be shown to be commutative by use of the canonical forms).

In the particular case  $w = w' = 0$ ,  $D$  and  $D'$  as well as  $G$  are harmonic biaxial homographies or line reflections. The axes of  $G$  are the lines joining the intersections of the axes of  $D$  with those of  $D'$  and are conjugate lines as to  $\Sigma$ , and indeed can by proper choice of the parameters be made any two conjugate lines as to  $\Sigma$ .

We may therefore state the Theorem:

**THEOREM X.** *If  $\Sigma$  is any non-singular quadric and if  $G$  is a line reflection whose axes are conjugate lines as to  $\Sigma$ , there exist two symmetrical triples of involutions  $A, B, C$  and  $A', B', C'$ , the first triple having as axes lines of one regulus on  $\Sigma$ , the second triple having as axes lines of the conjugate regulus, such that  $G$  is expressible in the form*

$$G = (xA + yB + zC)(x'A' + y'B' + z'C').$$

Again let  $n = 2$  so that  $A, B$  and  $C$  are involutions of points on a point row with double points

$$G_1 = B_{-1}, \quad B_1 = C_1, \quad C_1 = G_{-1},$$

$G_1$  and  $G_{-1}$  being the double points of  $A$ , etc. Theorem VII can then be given a geometric interpretation as follows:

**THEOREM XI.** *Let  $G, B$  and  $C$  be three points on a point row  $\ell$  and let  $U$  be any point of  $\ell$  distinct from these three. If then  $V$  is the fourth harmonic of  $U$  with respect to  $G$  and  $B$ ,  $W$  the fourth harmonic of  $V$  with respect to  $B$  and  $C$ , and  $U_1$  the fourth harmonic of  $W$  with respect to  $C$  and  $G$ , there are exactly two positions on  $\ell$  at which  $U$  can be taken so that  $U_1$  coincides with  $U$ .*

# THE GEOMETRY OF TENSORS OF THE FIRST ORDER

BY ARCHIBALD HENDERSON

*Department of Mathematics, University of North Carolina*

WITH SEVEN FIGURES\*

## INTRODUCTION

Among the many difficulties which constitute obstacles to a full understanding of the Generalized Theory of Relativity, not the least and perhaps the greatest is the essentially modern discipline of Tensor Analysis. The idea of a Vector was introduced into the literature by A. F. Möbius in 1827 in his famous work, *Der barycentrische Calcul*, and later found development in various directions at the hands of H. Grassmann in his *Ausdehnungslehre* and of Sir William Hamilton in his self-named quaternions. The utility of the concept of a vector, defined as a quantity having both magnitude and direction, has fully established itself within recent years, and has become recognized as an indispensable tool in many branches of pure and applied mathematics, theoretical physics, electricity and magnetism.

In 1898 W. Voigt, who has the honor of deriving the so-called Lorentz Transformation equations, which constituted the starting point of the Special Relativity Theory ("Ueber das Doeplersche Princip," *Goettinger Nachrichten*, 1887), first introduced the concept and name Tensor in the modern sense, from the geometric example of what we now call a mixed symmetric tensor representing the uniform stretching of a medium along three mutually perpendicular directions successively (Voigt, W., "Die fundamentalen Eigenschaften der Krystalle," Leipzig, 1898).

Three years later, the Italian mathematicians, Ricci and Levi-Civita, developed their powerful analysis known as "The Absolute Differential Calculus" (Ricci, G., and Levi-Civita, T., "Méthodes de Calcul différentiel absolu," *Mathematische Annalen*, 1901). They introduced a generalized form of vector, recognized as a tensor in the Voigt sense, arising from a specially defined differentiation of vectors. They introduced, however, many new types of tensor unsuspected by Voigt.

The famous paper of Einstein, written in collaboration with Grossmann, in which was developed in a simple and orderly manner the theory of tensor analysis, as the inevitable tool for Generalized Relativity Theory, marks the real beginning of the contemporary interest in and preoccupation with Tensor Analysis as a mathematical technic of surpassing power and elegance (Einstein, A., "Die Grundlage der allgemeinen Relativitaetstheorie," *Annalen der Physik*, 1916).

Tensors are usually recognized by means of a definition of covariancy, affording the transformation from one set of co-ordinate axes to another. Difficulties arise in effecting the transformation from a set of mutually rectangular axes to

\* For the drawings I am indebted to my colleague, Ralph M. Trimble.

any set, in the same dimensions, of curvilinear axes. A forthright and ingenious treatment of curvilinear co-ordinates in Euclidean space of three dimensions has recently been put forward by Paul Appell in the fifth volume of his great treatise on rational mechanics, written in collaboration with René Thiry (Appell, P., and Thiry, R., *Traité de Mécanique Rationnelle*, volume V: *Éléments de Calcul Tensorial*, 1926). This method is employed in the present paper.

Because of the abstractness of the subject and the dearth of illuminating illustrations, the writer has employed the new technic of Appell and Thiry for the purpose of exhibiting the subject in certain salient geometrical cases. These embrace the transformations in Euclidean space from three mutually perpendicular rectangular axes to the following: a) three mutually oblique axes; b) three mutually perpendicular axes, of which two are rectilinear and one curvilinear; c) three mutually perpendicular axes, of which one is rectilinear and two are curvilinear; d) three mutually perpendicular axes, of which all three are curvilinear. In a series of diagrams, the fundamental vectors and the covariant and contravariant components of tensors of the first order are exhibited. These results have been obtained after laborious calculations. The results, as shown in the diagrams, offer a complete explanation and exhibit of all the vectorial and tensorial qualities involved in these cases.

1. Choosing two consecutive points  $\bar{x}^i$  and  $\bar{x}^i + d\bar{x}^i$ , referred to three mutually rectangular axes  $OA^1, OA^2, OA^3$  in Euclidean space of three dimensions, we have, by the Pythagorean theorem

$$ds^2 = \bar{g}_{ik} d\bar{x}^i d\bar{x}^k \quad \left. \begin{array}{l} \\ \bar{g}_{ik} = \begin{cases} 0 & \text{if } i \neq k \\ 1 & \text{if } i = k \end{cases} \end{array} \right\} \quad (A)^*$$

with

This is the fundamental quadratic form, essentially invariant.

Now let us transform to a new set of three curvilinear axes, where  $\bar{x}^i = f^i(x^1, x^2, x^3)$  with the usual requirements of continuity and the assumption that  $\|\bar{x}_k^i\| \neq 0$ . We have

$$ds^2 = g_{rs} dx^r dx^s = \bar{g}_{rs} d\bar{x}^r d\bar{x}^s = \bar{g}_{rs} \bar{x}_r^s \bar{g}_{rs} dx^r dx^s,$$

where  $d\bar{x}^s = \bar{x}_r^s dx^r$ ,  $d\bar{x}^s = \bar{x}_s^r dx^s$ , designating  $\frac{\partial \bar{x}^i}{\partial x^r} = \bar{x}_r^i$ ,  $\frac{\partial x^i}{\partial \bar{x}^r} = x_r^i$ .

Hence we have the results for covariant tensors

$$g_{rs} = \bar{x}_r^s \bar{x}_s^r \bar{g}_{rs} = \bar{x}_r^s \bar{x}_s^r \dots \dots \dots \quad (1)_a$$

with similar results for contravariant and mixed tensors

$$g^{rs} = x_r^s x_s^r g^{rs} = x_r^s x_s^r \dots \dots \dots \quad (1)_b$$

\* Let us record here the following:

$$\bar{g} = \|\bar{g}_{ik}\| = 1, \bar{g}_k^i = g_k^i = \begin{cases} 0 & \text{if } i \neq k \\ 1 & \text{if } i = k \end{cases}$$

and

$$g_r^s = x_p^r x_s^i \bar{g}^{ri} = x_p^r x_s^i \quad \dots \dots (1).$$

and consequently

$$g_r^s = \begin{cases} 0 & \text{if } r \neq s \\ 1 & \text{if } r = s \end{cases}$$

2. Consider now the formulas of transformation  $\bar{x}^i = f^i(x^1, x^2, x^3)$ . If we let  $x^1$  vary,  $x^2$  and  $x^3$  remaining constant, the point  $M(x^1, x^2, x^3)$  will describe a curve, designated  $x^1$  = variable; and similarly for the other coordinates. If we let  $x^2$  and  $x^3$  vary simultaneously,  $x^1$  remaining fixed, we shall thereby obtain a surface containing the two curves  $x^2$  = variable,  $x^3$  = variable, passing through the point  $M$ , which we may call the surface  $x^1$  = constant; and similarly in the other cases. The curves and surfaces just described, which are associated with every point of space, are called the curves and surfaces of the co-ordinates.

By a proper orientation of the tangents, at  $M$  to the curves of the co-ordinates, forming a triheder,<sup>†</sup> we obtain three axes  $T^1, T^2, T^3$  passing through  $M$ . By suitably orienting the normals at  $M$  to the surfaces of co-ordinates, we obtain a second triheder  $N^1, N^2, N^3$ . By the usual method in differential geometry, using formulas (1) above, we have

$$\cos \widehat{A^r T^s} = \frac{\bar{x}_s^r}{\sqrt{g_{ss}}} \quad (\text{not summed, 9 in all}) \quad (2)$$

In a similar manner, employing conditions of orthogonality, we find

$$\cos \widehat{A^r N^s} = \frac{x_s^r}{\sqrt{g^{ss}}} \quad (\text{not summed, 9 in all}) \quad (3)$$

From equations (2) and (3), using the familiar formulas of differential geometry, we find

$$\cos \widehat{T^p T^q} = \frac{g_{pq}}{\sqrt{g_{pp}} \sqrt{g_{qq}}} \quad (\text{not summed}) \quad (4)$$

and

$$\cos N^p N^q = \frac{g^{pq}}{\sqrt{g^{pp}} \sqrt{g^{qq}}} \quad (\text{not summed}) \quad (5)$$

3. In the system of rectangular coordinates, the components of any vector  $MV$  may be regarded as either contravariant or covariant components of a tensor of the first order. These will be denoted indifferently as  $\xi^1, \xi^2, \xi^3$  or  $\xi_1, \xi_2, \xi_3$ . In the system of curvilinear co-ordinates, the same tensor will have

\* In a system of rectangular co-ordinates, no distinction can be drawn between covariant and contravariant components of the same tensor, since  $\bar{A}_r = \bar{g}_r, A^r = \bar{A}^r$ .

† Obviously the condition  $\|\bar{x}_h^i\| \neq 0$  assures a real triheder, the faces of which are the tangent planes to the surfaces of the co-ordinates

contravariant and covariant components, according to the rules of transformation of tensorial systems; but in this case the components of different types will not be identical.

The following definitions and notations are given, for conciseness in expression and elucidation of the diagrams (Fig. 3):

$Mt' = P_{rr}$ , the algebraic measure of the orthogonal projection of the vector  $MV$  upon the tangent  $T'$ ;

$Mn' = P_{Nr}$ , the algebraic measure of the orthogonal projection of the vector  $MV$  upon the normal  $N'$ ;

$M\tau' = C_{rr}$ , the algebraic measure of the component along  $T'$  of the vector  $MV$  decomposed along the three tangents;

$M\nu' = C_{Nr}$ , the algebraic measure of the component along  $N'$  of the vector  $MV$  decomposed along the three normals.

From the theory of projections, we have

$$\left. \begin{aligned} P_{rr} &= \sum_i \xi_i \cos \widehat{A^i T'} = \sum_i \frac{\xi_i \bar{x}_r^i}{\sqrt{g_{rr}}} \\ P_{Nr} &= \sum_i \xi_i \cos \widehat{A^i N'} = \sum_i \frac{\xi_i \bar{x}_r^i}{\sqrt{g^{rr}}} \end{aligned} \right\} \text{(not summed as to } r\text{)} \\ \xi^i = \sum_r C_{rr} \cos \widehat{A^i T'} = \sum_r \frac{C_{rr} \bar{x}_r^i}{\sqrt{g_{rr}}} \\ \xi_i = \sum_r C_{Nr} \cos \widehat{A^i N'} = \sum_r \frac{C_{Nr} \bar{x}_r^i}{\sqrt{g^{rr}}}$$

On the other hand, according to the laws of transformation of tensorial systems, we have

$$\begin{aligned} \xi_r &= \sum_i \xi_i \bar{x}_r^i \quad \text{and} \quad \xi^i = \sum_r \xi_r \bar{x}_r^i \\ \xi^r &= \sum_i \xi^i \bar{x}_i^r \quad \text{and} \quad \xi_i = \sum_r \xi_r \bar{x}_i^r. \end{aligned}$$

Hence, by comparison of the preceding formulas, we have

$$\left. \begin{aligned} P_{rr} &= \frac{\xi_r}{\sqrt{g_{rr}}}, \quad C_{rr} = \sqrt{g_{rr}} \xi^r \\ P_{Nr} &= \frac{\xi^r}{\sqrt{g^{rr}}}, \quad C_{Nr} = \sqrt{g^{rr}} \xi_r \end{aligned} \right\} \text{(not summed) . . . . . (6)}$$

In the event that the curvilinear axes are orthogonal, the  $(ds)^2$  contains only square terms, and the formulas (6) reduce to a single formula

$$\left. \begin{aligned} \xi_r &= g_{rr} \xi^r, \quad \text{or} \quad \xi^r = g^{rr} \xi_r \\ g^{rr} &= \frac{1}{g_{rr}} \end{aligned} \right\} \text{(not summed) . . . . . (6')}$$

In orthogonal cartesian co-ordinates, the length  $l$  of the vector  $MV$  is given by the formula

$$l^2 = (\xi^1)^2 + (\xi^2)^2 + (\xi^3)^2 = g_{ik}\xi^i\xi^k$$

Since, in the second form, the tensorial character of an invariant is indicated by the second member, we may also write

In the subsequent work, the familiar notations are employed:

$$g = \begin{vmatrix} g_{11} & g_{12} & g_{13} \\ g_{21} & g_{22} & g_{23} \\ g_{31} & g_{32} & g_{33} \end{vmatrix}, \quad g^{ik} = \frac{\text{Co-factor of } g_{ik}}{g}$$

## *Applications*

4. TRANSFORMATION TO OBLIQUE CO-ORDINATES. The formulas of transformation from a rectilinear set of three mutually perpendicular axes ( $\bar{x}$ ) to a rectilinear set of three oblique axes ( $x$ ), keeping  $\bar{x}^1$  and  $x^1$  coincident, are easily seen to be

$$\left. \begin{aligned} \bar{x}^1 &= x^1 + \nu x^2 + \mu x^3 \\ \bar{x}^2 &= \sqrt{1 - \nu^2} x^2 + \frac{\lambda - \mu \nu}{\sqrt{1 - \nu^2}} x^3 \\ \bar{x}^3 &= \frac{\sqrt{\nu}}{\sqrt{1 - \nu^2}} x^3 \end{aligned} \right\} \text{or for infinitesimal transformation} \\ d\bar{x}^1 &= dx^1 + \nu dx^2 + \mu dx^3, \text{ etc.} \end{aligned}$$

where  $\lambda, \mu, \nu = \cos \phi_1, \cos \phi_2, \cos \phi_3$  and

$$g = \begin{vmatrix} 1 & \nu & \mu \\ \nu & 1 & \lambda \\ \mu & \lambda & 1 \end{vmatrix} = 1 - \lambda^2 - \mu^2 - \nu^2 + 2\lambda\mu\nu \quad (\text{See Fig. 1})$$

Hence

$$(ds)^2 = (d\bar{x}^1)^2 + (d\bar{x}^2)^2 + (d\bar{x}^3)^2 = (dx^1)^2 + (dx^2)^2 + (dx^3)^2 + 2\lambda dx^2 dx^3 + 2\mu dx^3 dx^1 + 2\nu dx^1 dx^2.$$

The value of  $g$  is identified to be the square of the volume of the unit parallelopiped with its edges along the three oblique axes. Now the volume of the same parallelopiped =  $\{(1)(1) \sin \phi_3\} \cos \theta_3$ ; and

$$\begin{aligned}\therefore \sqrt{g} &= \sin \phi_1 \cos \theta_1 = \sin \phi_2 \cos \theta_2 = \sin \phi_3 \cos \theta_3 \\ &= \sqrt{1 - \lambda^2} \cos \theta_1 = \sqrt{1 - \mu^2} \cos \theta_2 = \sqrt{1 - \nu^2} \cos \theta_3\end{aligned}$$

where  $\theta_1, \theta_2, \theta_3$  are the angles between the co-ordinate axes  $x^1, x^2, x^3$  and the normals to the co-ordinate planes  $x^1 = \text{constant}$ ,  $x^2 = \text{constant}$ ,  $x^3 = \text{constant}$  (Fig. 2).

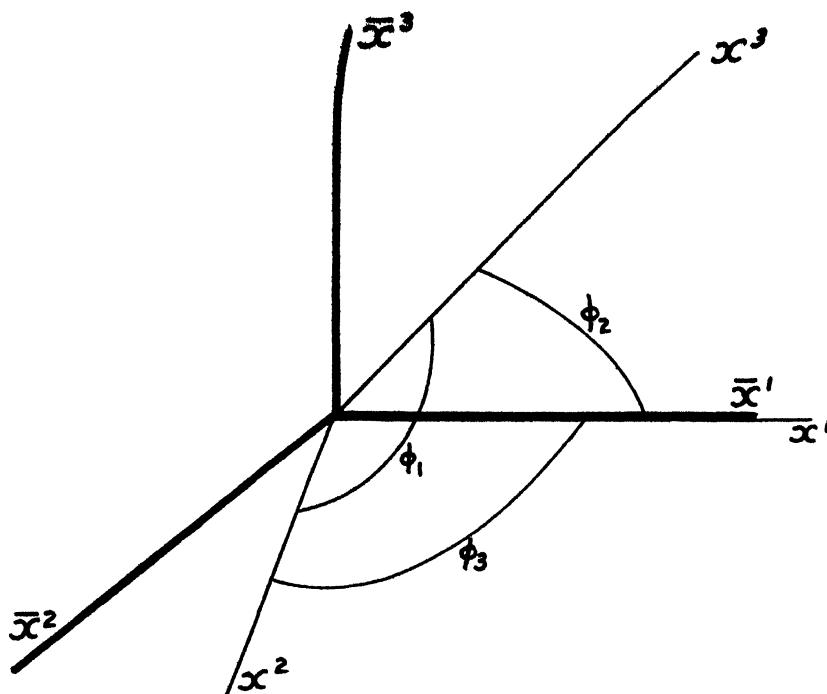


FIG. 1

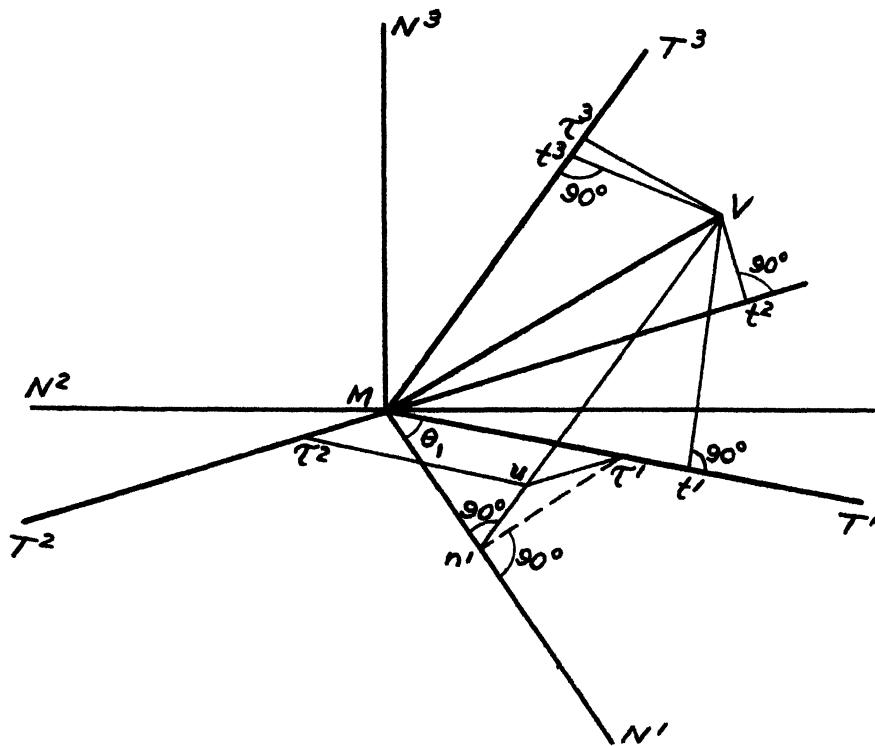


FIG. 2

Here  $g_{11} = g_{22} = g_{33} = 1$ ,  $g_{23} = \lambda$ ,  $g_{31} = \mu$ ,  $g_{12} = \nu$

$$g^{11} = \frac{1 - \lambda^2}{g} = \sec^2 \theta_1, \quad g^{22} = \frac{1 - \mu^2}{g} = \sec^2 \theta_2, \quad g^{33} = \frac{1 - \nu^2}{g} = \sec^2 \theta_3$$

$$\text{Also } \xi_1 = P_{T^1} = Mt^1, \quad \xi_2 = P_{T^2} = Mt^2, \quad \xi_3 = P_{T^3} = Mt^3$$

$$\xi^1 = \sec \theta_1 \cdot Mn^1, \quad \xi^2 = \sec \theta_2 \cdot Mn^2, \quad \xi^3 = \sec \theta_3 \cdot Mn^3.$$

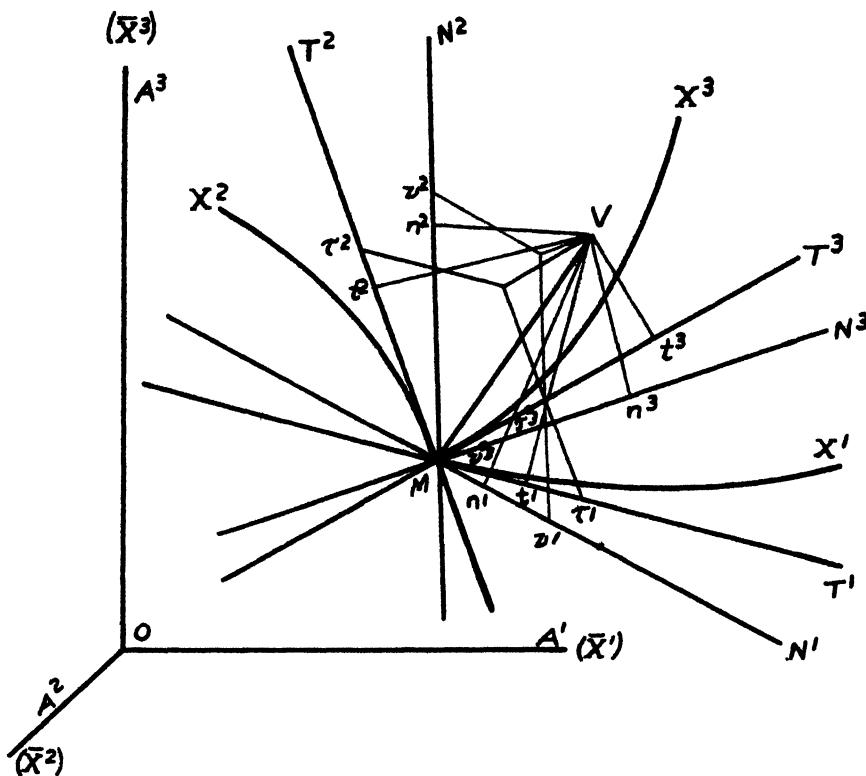


FIG. 3

Now through  $n^1$ ,  $\perp$  to  $Mn^1$  in the plane  $N^1MT^1$ , draw a line meeting the line  $MT^1$  in the point  $\tau^1$ . Then plane  $Vn^1\tau^1$  will be  $\perp$  to line  $MN^1$  and hence  $\parallel$  to plane  $T^2MT^3$ . Hence  $u\tau^1$ , the intersection of plane  $Vn^1\tau^1$  with plane  $T^1MT^2$ , will be  $\parallel$  to  $MT^2$ . Hence  $M\tau^1, \tau^1u, uV$  are the three components of  $MV$  in the directions of the  $x^i$ . They are shown on the axes as  $M\tau^1, M\tau^2, M\tau^3$ . Hence  $M\tau^1 = \xi^1, M\tau^2 = \xi^2, M\tau^3 = \xi^3$ .

Next project successively  $MV$  and these components along  $x^i$ . Then

$$\xi_1 = \xi^1 + \nu\xi^2 + \mu\xi^3$$

$$\xi_2 = \nu\xi^1 + \xi^2 + \lambda\xi^3$$

$$\xi_3 = \mu\xi^1 + \lambda\xi^2 + \xi^3$$

The tensors  $\xi_1, \xi^1$  are called reciprocal with respect to the quadratic differential form  $(ds)^2$ . Solving these equations for  $\xi^1, \xi^2, \xi^3$ , we have

$$\begin{aligned}\xi^1 &= \frac{1 - \lambda^2}{g} \xi_1 + \frac{\lambda\mu - \nu}{g} \xi_2 + \frac{\nu\lambda - \mu}{g} \xi_3 \\ \xi^2 &= \frac{\lambda\mu - \nu}{g} \xi_1 + \frac{1 - \mu^2}{g} \xi_2 + \frac{\mu\nu - \lambda}{g} \xi_3 \\ \xi^3 &= \frac{\nu\lambda - \mu}{g} \xi_1 + \frac{\mu\nu - \lambda}{g} \xi_2 + \frac{1 - \nu^2}{g} \xi_3\end{aligned}$$

Also we have from (6),

$$Mn^1 = \frac{\xi^1}{\sqrt{\frac{1 - \lambda^2}{g}}}, \quad Mn^2 = \frac{\xi^2}{\sqrt{\frac{1 - \mu^2}{g}}}, \quad Mn^3 = \frac{\xi^3}{\sqrt{\frac{1 - \nu^2}{g}}}$$

and

$$M\nu^1 = \sqrt{\frac{1 - \lambda^2}{g}} \xi_1, \quad M\nu^2 = \sqrt{\frac{1 - \mu^2}{g}} \xi_2, \quad M\nu^3 = \sqrt{\frac{1 - \nu^2}{g}} \xi_3$$

Employing the inverse transformation

$$\begin{aligned}x^1 &= \bar{x}^1 - \frac{\nu}{\sqrt{1 - \nu^2}} \bar{x}^2 + \frac{\nu\lambda - \mu}{\sqrt{(1 - \nu^2)g}} \bar{x}^3 \\ x^2 &= \frac{1}{\sqrt{1 - \nu^2}} \bar{x}^2 + \frac{\mu\nu - \lambda}{\sqrt{(1 - \nu^2)g}} \bar{x}^3 \\ x^3 &= \frac{\sqrt{1 - \nu^2}}{\sqrt{g}} \bar{x}^3\end{aligned}$$

where  $g = 1 - \lambda^2 - \mu^2 - \nu^2 + 2\lambda\mu\nu$  we find from the laws of transformation of tensorial systems

$$\left. \begin{aligned}\xi_1 &= \bar{\xi}_1 \\ \xi_2 &= \nu\bar{\xi}_1 + \sqrt{1 - \nu^2} \bar{\xi}_2 \\ \xi_3 &= \mu\bar{\xi}_1 + \frac{\lambda - \mu\nu}{\sqrt{1 - \nu^2}} \bar{\xi}_2 + \frac{\sqrt{g}}{\sqrt{1 - \nu^2}} \bar{\xi}_3\end{aligned}\right\}$$

and

$$\begin{aligned}\xi^1 &= \bar{\xi}^1 - \frac{\nu}{\sqrt{1 - \nu^2}} \bar{\xi}^2 + \frac{\nu\lambda - \mu}{\sqrt{(1 - \nu^2)g}} \bar{\xi}^3 \\ \xi^2 &= \frac{1}{\sqrt{1 - \nu^2}} \bar{\xi}^2 + \frac{\mu\nu - \lambda}{\sqrt{(1 - \nu^2)g}} \bar{\xi}^3 \\ \xi^3 &= \frac{\sqrt{1 - \nu^2}}{\sqrt{g}} \bar{\xi}^3\end{aligned}$$

Since  $dx^1 = \xi^1$ ,  $dx^2 = \xi^2$ ,  $dx^3 = \xi^3$ , we find that

$$(MV)^2 = (ds)^2 = (\xi^1)^2 + (\xi^2)^2 + (\xi^3)^2 + 2\lambda\xi^2\xi^3 + 2\mu\xi^3\xi^1 + 2\nu\xi^1\xi^2.$$

Other expressions for  $(ds)^2$  in terms of  $\xi^1$ ,  $\xi^2$ , and  $\xi^3$  may also be found, from the equations developed above.

5. TRANSFORMATION TO CYLINDRICAL CO-ORDINATES. The transformation from a set of three mutually perpendicular axes ( $\bar{x}$ ) to a set of three cylindrical axes ( $T^i$ ) is (Fig. 4)

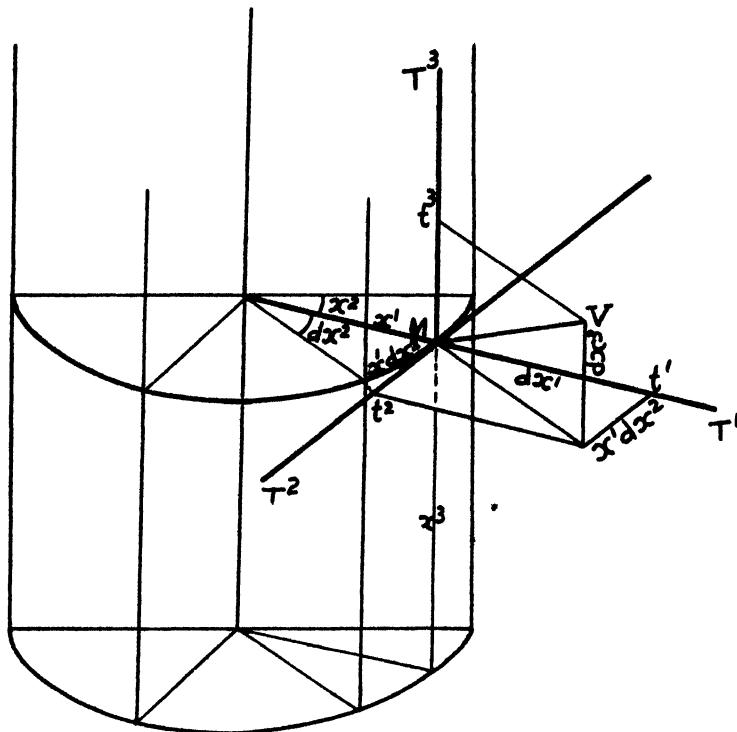


FIG. 4

$$\begin{aligned} \bar{x}^1 &= x^1 \cos x^2 \\ \bar{x}^2 &= x^1 \sin x^2 \\ \bar{x}^3 &= x^3 \end{aligned} \quad \left. \begin{aligned} \text{where } (ds)^2 &= (d\bar{x}^1)^2 + (d\bar{x}^2)^2 + (d\bar{x}^3)^2 \end{aligned} \right\}$$

Now

$$d\bar{x}^1 = \cos x^2 dx^1 - x^1 \sin x^2 dx^2$$

$$d\bar{x}^2 = \sin x^2 dx^1 + x^1 \cos x^2 dx^2$$

$$d\bar{x}^3 = dx^3$$

$$\therefore (ds)^2 = (d\bar{x}^1)^2 + (d\bar{x}^2)^2 + (d\bar{x}^3)^2 = (dx^1)^2 + (x^1)^2(dx^2)^2 + (dx^3)^2 \text{ and } g_{11} = 1, \\ g_{22} = (x^1)^2, g_{33} = 1, g^{11} = 1, g^{22} = \frac{1}{(x^1)^2}, g^{33} = 1.$$

Now by (6)  $P_{T1} = Mt^1 = \xi_1$ ,  $P_{T2} = Mt^2 = \frac{1}{x^1} \xi_2$ ,  $P_{T3} = Mt^3 = \xi_3$  and  $P_{N1} = Mn^1 = \xi^1$ ,  $P_{N2} = Mn^2 = x^1 \xi^2$ ,  $P_{N3} = Mn^3 = \xi^3$ .

For this case of orthogonal axes,  $\sqrt{g^{rr}} \xi_r = \sqrt{g_{rr}} \xi^r$ , and therefore

$$\xi_1 = \xi^1, \frac{1}{x^1} \xi_2 = x^1 \xi^2, \xi_3 = \xi^3, \text{ giving } \xi_1 = \xi^1, \xi_2 = (x^1)^3 \xi^2, \xi_3 = \xi^3.$$

From the formulas  $P_{T1} = \frac{\sum_i \xi_i \bar{x}_i^1}{\sqrt{g_{rr}}}$ ,  $P_{N1} = \frac{\sum_i \xi_i x_i^1}{\sqrt{g^{rr}}}$ , we have

$$\xi_1 = (\cos x^2) \bar{\xi}_1 + (\sin x^2) \bar{\xi}_2$$

$$\xi_2 = (-x^1 \sin x^2) \bar{\xi}_1 + (x^1 \cos x^2) \bar{\xi}_2$$

$$\xi_3 = \bar{\xi}_3$$

and

$$\xi^1 = (\cos x^2) \bar{\xi}_1 + (\sin x^2) \bar{\xi}_2$$

$$\xi^2 = \left( -\frac{1}{x^1} \sin x^2 \right) \bar{\xi}_1 + \left( \frac{1}{x^1} \cos x^2 \right) \bar{\xi}_2$$

$$\xi^3 = \bar{\xi}_3$$

From  $(MV)^2 = g_{kk} \xi^k \xi^k = g^{kk} \xi_k \xi_k = \xi^k \xi_k$ , we have

$$(ds)^2 = (\xi^1)^2 + (x^1)^2 (\xi^2)^2 + \xi^3 = \xi_1^2 + \left( \frac{1}{(x^1)^2} \right) \xi_2^2 + \xi_3^2 = \xi^1 \xi_1 + \xi^2 \xi_2 + \xi^3 \xi_3.$$

6. TRANSFORMATION TO SPHERICAL CO-ORDINATES. A transformation from a set of three mutually perpendicular axes ( $\bar{x}$ ) to a set of three spherical axes ( $T^i$ ) is (Fig. 5)

$$\left. \begin{aligned} \bar{x}^1 &= x^1 \sin x^2 \cos x^3 \\ \bar{x}^2 &= x^1 \sin x^2 \sin x^3 \\ \bar{x}^3 &= x^1 \cos x^2 \end{aligned} \right\} \text{ where } (ds)^2 = (dx^1)^2 + (dx^2)^2 + (dx^3)^2$$

$$\text{Now } d\bar{x}^1 = \sin x^2 \cos x^3 dx^1 + x^1 \cos x^2 \cos x^3 dx^2 - x^1 \sin x^2 \sin x^3 dx^3$$

$$d\bar{x}^2 = \sin x^2 \sin x^3 dx^1 + x^1 \cos x^2 \sin x^3 dx^2 + x^1 \sin x^2 \cos x^3 dx^3$$

$$d\bar{x}^3 = \cos x^2 dx^1 - x^1 \sin x^2 dx^2$$

$$\therefore (ds)^2 = (dx^1)^2 + (x^1)^2 (dx^2)^2 + (x^1 \sin x^2)^2 (dx^3)^2, \text{ with}$$

$$g_{11} = 1, \quad g_{22} = (x^1)^2, \quad g_{33} = (x^1)^2 (\sin x^2)^2$$

and

$$g^{11} = 1, \quad g^{22} = \frac{1}{(x^1)^2}, \quad g^{33} = \frac{1}{(x^1)^2 (\sin x^2)^2}$$

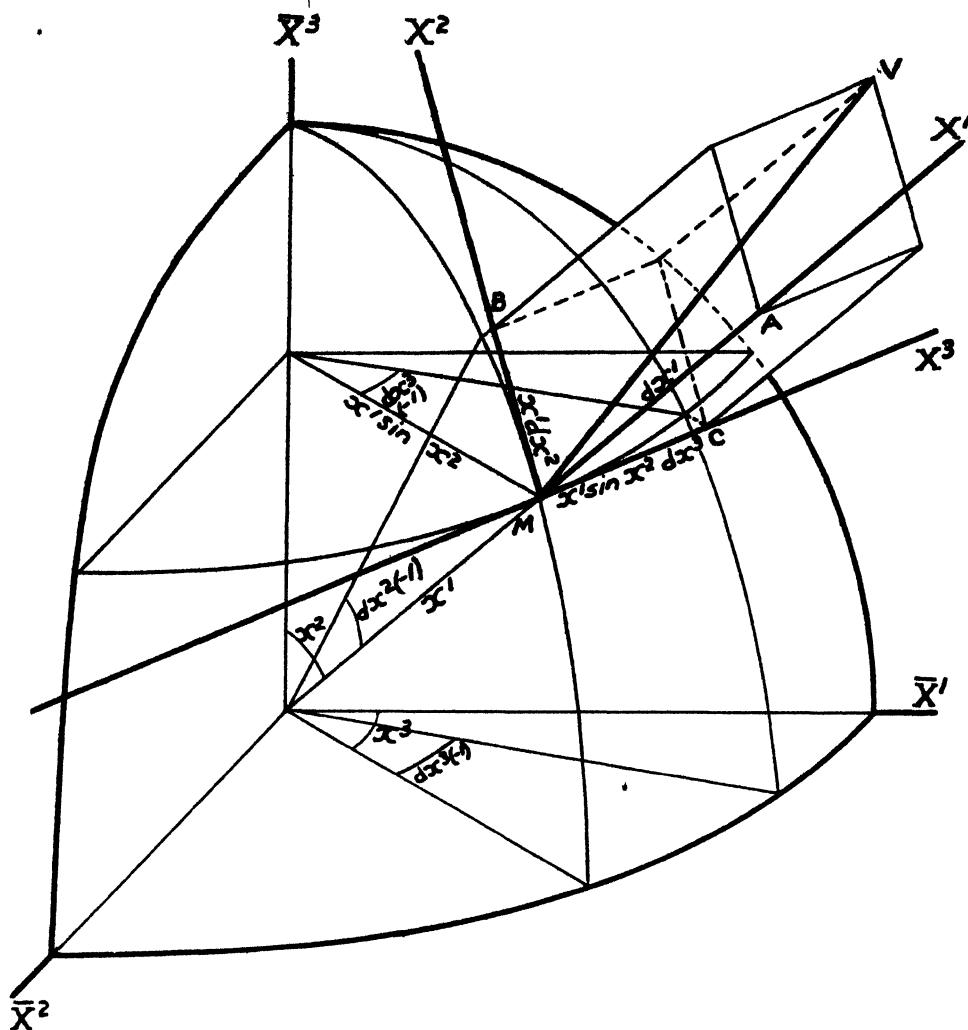


FIG. 5

Now

$$\begin{aligned}
 J = & \begin{vmatrix}
 \bar{x}_1^1 & \bar{x}_2^1 & \bar{x}_3^1 \\
 \bar{x}_1^2 & \bar{x}_2^2 & \bar{x}_3^2 \\
 \bar{x}_1^3 & \bar{x}_2^3 & \bar{x}_3^3
 \end{vmatrix} \\
 & \sin x^2 \cos x^3, \quad x^1 \cos x^2 \cos x^3, \quad -x^1 \sin x^3 \sin x^3! \\
 & \sin x^2 \sin x^3, \quad x^1 \cos x^2 \sin x^3, \quad x^1 \sin x^2 \cos x^3 = (x^1)^2 (\sin x^2), \\
 & \cos x^2, \quad -x^1 \sin x^2, \quad 0
 \end{aligned}$$

which is  $\neq 0$ , except for  $x^1 = 0$ ,  $\sin x^2$ , giving singular points on the polar axis:  $x^1 = 0$ ,  $x^2 = 0$  or  $\pi$ . Hence we will have a genuine triheder with edges tangent to the three space curves  $x^1 = \text{variable}$ ,  $x^2 = \text{variable}$ ,  $x^3 = \text{variable}$ .

Now from  $\xi_r = \bar{x}_r^i \xi_i$  and  $\xi^r = \bar{x}_i^r \xi^i$ , we have

$$\begin{aligned}\xi_1 &= \sin x^3 \cos x^3 \cdot \xi_1 + \sin x^2 \sin x^3 \cdot \xi_2 + \cos x^3 \cdot \xi_3 \\ \xi_2 &= x^1 \cdot [\cos x^3 \cos x^3 \cdot \xi_1 + \cos x^3 \sin x^3 \cdot \xi_2 - \sin x^3 \cdot \xi_3] \\ \xi_3 &= x^1 \sin x^3 [-\sin x^3 \cdot \xi_1 + \cos x^3 \xi^2] \\ \xi^1 &= \sin x^3 \cos x^3 \xi^1 + \sin x^2 \cos x^3 \xi^2 + \cos x^3 \xi^3 \\ \xi^2 &= \frac{1}{x^1} [\cos x^3 \cos x^3 \xi^1 + \cos x^2 \sin x^3 \xi^2 - \sin x^3 \xi^3] \\ \xi^3 &= \frac{1}{x^1 \sin x^3} [(-\sin x^3) \xi^1 + (\cos x^3) \xi^2]\end{aligned}$$

Here we note, for verification, from (6') that  $\xi_1 = \xi^1$ ,  $\xi_2 = (x^1)^2 \xi^2$ ,  $\xi_3 = (x^1 \sin x^3)^2 \xi^3$  and  $g^{rr} = \frac{1}{g_{rr}}$ . Also

$$\begin{aligned}(MV)^2 &= g_{ik} \xi^i \xi^k = (\xi^1)^2 + (x^1)^2 (\xi^2)^2 + (x^1 \sin x^3)^2 (\xi^3)^2 \\ &= g^{kk} \xi_k \xi_k = (\xi_1)^2 + \frac{1}{(x^1)^2} (\xi_2)^2 + \frac{1}{(x^1 \sin x^3)^2} (\xi_3)^2 \\ &= \xi^1 \xi_1 + \xi^2 \xi_2 + \xi^3 \xi_3\end{aligned}$$

7. A SYSTEM OF THREE CONFOCAL CONICOIDS. Given a system of three confocal conicoids

$$\left. \begin{array}{l} (1) \quad \frac{\bar{x}^{1^2}}{x^{1^2}} + \frac{\bar{x}^{2^2}}{x^{1^2} - b^2} + \frac{\bar{x}^{3^2}}{x^{1^2} - c^2} = 1 \quad (S_1) \\ (2) \quad \frac{\bar{x}^{1^2}}{x^{2^2}} + \frac{\bar{x}^{2^2}}{x^{2^2} - b^2} + \frac{\bar{x}^{3^2}}{x^{2^2} - c^2} = 1 \quad (S_2) \\ (3) \quad \frac{\bar{x}^{1^2}}{x^{3^2}} + \frac{\bar{x}^{2^2}}{x^{3^2} - b^2} + \frac{\bar{x}^{3^2}}{x^{3^2} - c^2} = 1 \quad (S_3) \end{array} \right\} \begin{array}{l} (x^{1^2} > c^2 > b^2) \\ \text{where } (b^2 < x^{2^2} < c^2) \\ (0 < x^{3^2} < b^2) \end{array}$$

namely an ellipsoid ( $S_1$ ), an hyperboloid of one sheet ( $S_2$ ), and an hyperboloid of two sheets ( $S_3$ ) (Fig. 6).

Solving (1), (2), and (3) by Kramer's rule, we find the familiar expressions known as Lamé's co-ordinates:

$$\left. \begin{array}{l} (4) \quad \bar{x}^{1^2} = \frac{x^{1^2} x^{2^2} x^{3^2}}{b^2 c^2}, \quad \bar{x}^{2^2} = \frac{(x^{1^2} - b^2)(x^{2^2} - b^2)(x^{3^2} - b^2)}{b^2 (b^2 - c^2)}, \\ \bar{x}^{3^2} = \frac{(x^{1^2} - c^2)(x^{2^2} - c^2)(x^{3^2} - c^2)}{c^2 (c^2 - b^2)} \end{array} \right.$$

Taking logarithms of equations (4) and differentiating, we find:

$$(5) \quad d\bar{x}^1 = \frac{\bar{x}^1 dx^1}{x^1} + \frac{\bar{x}^1 dx^2}{x^2} + \frac{\bar{x}^1 dx^3}{x^3}$$

## GEOMETRY OF TENSORS OF FIRST ORDER

$$(6) \quad d\bar{x}^2 = \frac{\bar{x}^2 x^1 dx^1}{x^{1^2} - b^2} + \frac{\bar{x}^2 x^2 dx^2}{x^{2^2} - b^2} + \frac{\bar{x}^2 x^3 dx^3}{x^{3^2} - b^2}$$

(6)

$$(7) \quad d\bar{x}^3 = \frac{\bar{x}^3 x^1 dx^1}{x^{1^2} - c^2} + \frac{\bar{x}^3 x^2 dx^2}{x^{2^2} - c^2} + \frac{\bar{x}^3 x^3 dx^3}{x^{3^2} - c^2}$$

(7)

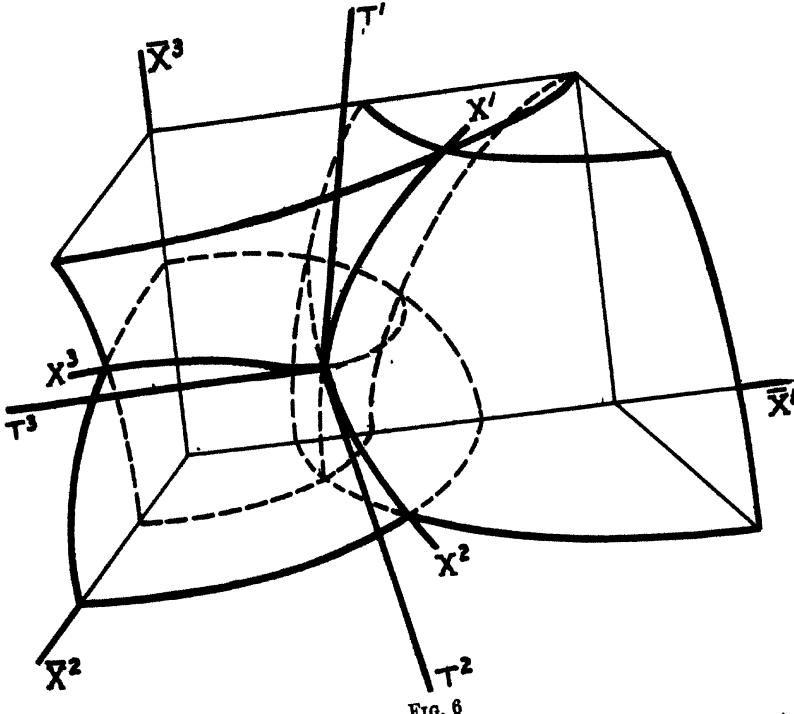


FIG. 6

Squaring and adding (5), (6), and (7), and making use of (1), (2), and (3), we find after laborious calculations

$$(8) \quad (ds)^2 = (dx^1)^2 + (dx^2)^2 + (dx^3)^2 = \frac{(x^{1^2} - x^{2^2})(x^{1^2} - x^{3^2})}{(x^{1^2} - b^2)(x^{1^2} - c^2)} (dx^1)^2 + \frac{(x^{2^2} - x^{3^2})(x^{2^2} - x^{1^2})}{(x^{2^2} - b^2)(x^{2^2} - c^2)} (dx^2)^2 + \frac{(x^{3^2} - x^{1^2})(x^{3^2} - x^{2^2})}{(x^{3^2} - b^2)(x^{3^2} - c^2)} (dx^3)^2$$

where  $ds$  denotes the distance between the points  $x^i$  and  $x^i + dx^i$ .

Here  $x^1, x^2, x^3$  are called elliptic co-ordinates; and when they are given, we may suppose the surfaces  $S_1, S_2, S_3$  constructed, and their common points  $(\bar{x}^1, \bar{x}^2, \bar{x}^3)$  known from (4).

If we set  $dx^1 = 0$  in (8), then the two points lie in the surface  $S_1$ , and the formula for the distance between them ( $ds$ ) is

$$(ds)^2 = \frac{(x^{2^2} - x^{3^2})(x^{2^2} - x^{1^2})}{(x^{2^2} - b^2)(x^{2^2} - c^2)} (dx^2)^2 + \frac{(x^{3^2} - x^{1^2})(x^{3^2} - x^{2^2})}{(x^{3^2} - b^2)(x^{3^2} - c^2)} (dx^3)^2$$

If we set  $dx^3 = 0$  and  $dx^3 = 0$  in (8), then the two points lie in both the surfaces  $S_2$  and  $S_3$ , and we have

$$(ds)^2 = \frac{(x^{12} - x^{22})(x^{12} - x^{32})}{(x^{12} - b^2)(x^{12} - c^2)} (dx^1)^2$$

Since if  $dx^1, dx^2, dx^3$  are all zero,  $dx^1, dx^2, dx^3$  are all zero, from (5), (6) and (7), then  $dx^1, dx^2, dx^3$  are infinitesimal quantities measured in the lines of intersection of  $S_2, S_3$ ;  $S_2, S_1$ ;  $S_1, S_3$  respectively. These three curved lines are mutually perpendicular, as may be readily shown.

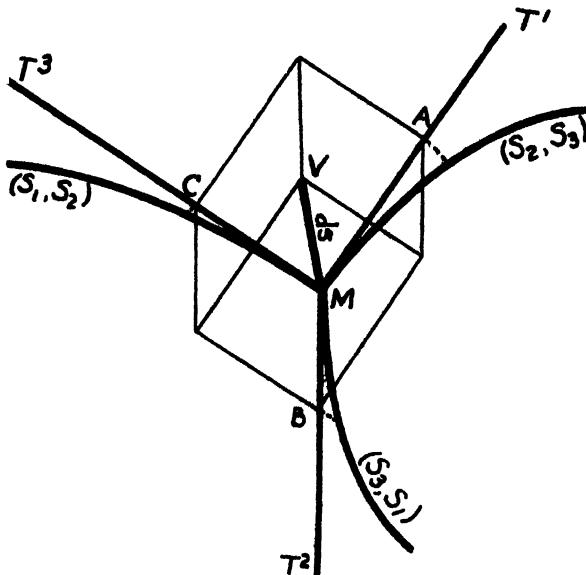


FIG. 7

Hence we may lay off, along the three tangents,  $MT^1, MT^2, MT^3$  the components  $MA, MB, MC$  of the vector  $MV$ . From the equations

$$MA = \sqrt{\frac{(x^{12} - x^{22})(x^{12} - x^{32})}{(x^{12} - b^2)(x^{12} - c^2)}} \cdot dx^1$$

$$MB = \sqrt{\frac{(x^{22} - x^{32})(x^{22} - x^{12})}{(x^{22} - b^2)(x^{22} - c^2)}} \cdot dx^2$$

$$MC = \sqrt{\frac{(x^{32} - x^{12})(x^{32} - x^{22})}{(x^{32} - b^2)(x^{32} - c^2)}} \cdot dx^3$$

we then determine  $dx^1, dx^2, dx^3$ , and lay them off along the three curvilinear axes. These quantities  $dx^1, dx^2, dx^3$  are the distances from the surfaces  $S_1, S_2, S_3$  to the consecutive surfaces corresponding to the point  $x^1 + dx^1, x^2 + dx^2, x^3 + dx^3$ .

Then

$$(MV)^2 = (MA)^2 + (MB)^2 + (MC)^2$$

where  $MA, MB, MC$  are the edges of a rectangular parallelopiped, of which  $MV$  is the diagonal.

Here

$$\begin{aligned} g_{11} &= \frac{(x^{12} - x^{32})(x^{12} - x^{22})}{(x^{12} - b^2)(x^{12} - c^2)}, & g_{22} &= \frac{(x^{22} - x^{32})(x^{22} - x^{12})}{(x^{22} - b^2)(x^{22} - c^2)}, \\ g_{33} &= \frac{(x^{32} - x^{12})(x^{32} - x^{22})}{(x^{32} - b^2)(x^{32} - c^2)}, \\ g^{11} &= \frac{(x^{12} - b^2)(x^{12} - c^2)}{(x^{12} - x^{32})(x^{12} - x^{22})}, & g^{22} &= \frac{(x^{22} - b^2)(x^{22} - c^2)}{(x^{22} - x^{32})(x^{22} - x^{12})}, \\ g^{33} &= \frac{(x^{32} - b^2)(x^{32} - c^2)}{(x^{32} - x^{12})(x^{32} - x^{22})}. \end{aligned}$$

From the formulas  $P_{T^1} = Mt^1 = \frac{\xi_1}{\sqrt{g_{11}}}$ ,  $P_{T^2} = Mt^2 = \frac{\xi_2}{\sqrt{g_{22}}}$ ,  $P_{T^3} = Mt^3 = \frac{\xi_3}{\sqrt{g_{33}}}$ , and  $P_{Tr} = \frac{\sum_i \xi_i \bar{x}_i}{\sqrt{g_{rr}}}$ , we find

$$\begin{aligned} \xi_1 &= \frac{x^2 x^3}{bc} \bar{\xi}_1 + \frac{x^1 \sqrt{(x^{22} - b^2)(x^{32} - b^2)}}{b \sqrt{(b^2 - c^2)(x^{12} - b^2)}} \bar{\xi}_2 + \frac{x^1 \sqrt{(x^{22} - c^2)(x^{32} - c^2)}}{c \sqrt{(c^2 - b^2)(x^{12} - c^2)}} \bar{\xi}_3 \\ \xi_2 &= \frac{x^3 x^1}{bc} \bar{\xi}_1 + \frac{x^2 \sqrt{(x^{12} - b^2)(x^{32} - b^2)}}{b \sqrt{(b^2 - c^2)(x^{22} - b^2)}} \bar{\xi}_2 + \frac{x^2 \sqrt{(x^{12} - c^2)(x^{32} - c^2)}}{c \sqrt{(c^2 - b^2)(x^{22} - c^2)}} \bar{\xi}_3 \\ \xi_3 &= \frac{x^1 x^2}{bc} \bar{\xi}_1 + \frac{x^3 \sqrt{(x^{12} - b^2)(x^{22} - b^2)}}{b \sqrt{(b^2 - c^2)(x^{32} - b^2)}} \bar{\xi}_2 + \frac{x^3 \sqrt{(x^{12} - c^2)(x^{22} - c^2)}}{c \sqrt{(c^2 - b^2)(x^{32} - c^2)}} \bar{\xi}_3 \end{aligned}$$

From the formulas  $\xi' = g^{rr} \xi_r$ , we have

$$\begin{aligned} \xi^1 &= \frac{(x^{12} - b^2)(x^{12} - c^2)}{(x^{12} - x^{32})(x^{12} - x^{22})} \bar{\xi}_1, & \xi^2 &= \frac{(x^{22} - b^2)(x^{22} - c^2)}{(x^{22} - x^{32})(x^{22} - x^{12})} \bar{\xi}_2, \\ \xi^3 &= \frac{(x^{32} - b^2)(x^{32} - c^2)}{(x^{32} - x^{12})(x^{32} - x^{22})} \bar{\xi}_3 \end{aligned}$$

and so we may write down also the corresponding formulas for  $\xi^1, \xi^2$ , and  $\xi^3$  in terms of  $\bar{\xi}_1, \bar{\xi}_2$ , and  $\bar{\xi}_3$ .

# ON THE EQUATIONS OF CERTAIN OSCULANTS

BY J. W. LASLEY, JR.

*Department of Mathematics, University of North Carolina*

## INTRODUCTION

In the analytic geometry of Descartes and Fermat as formulated in the seventeenth century, the relation between curve and function became for the first time apparent. Newton and Leibniz supplied the determination of direction by means of the derivative. They supplied also the determination of area by means of the integral. Newton soon formulated the notion of curvature, although Huygens<sup>1</sup> had preceded him in envisioning this concept. It remained for Abel Transon<sup>2</sup> in 1841 to provide the image of the third and fourth derivatives by means of his theory of aberrancy. These beautiful researches of Transon served as a point of departure for sporadic investigations into the general problem of osculants. Perhaps Cesaro<sup>3</sup> more than any other writer up to his time appreciated what Transon had done, but Cesaro made hardly a beginning of a theory of osculants. American mathematicians seemed little aware of the significance of these ideas until Wilczynski<sup>4</sup> recalled them to life with such force in his retiring address of 1916. In this paper Wilczynski asserted that "the notions, osculant and penosculant, are the fundamental concepts of differential geometry." He recounted the developments of Transon and Cesaro, but regretted the fact "that so many problems have remained untouched." Relative to certain osculants of plane curves, he stated, "I actually have most of these equations at my disposal."

So far as this writer can ascertain, Wilczynski did not in any of his papers make these equations available. Nor does he seem aware that in 1908, eight years earlier, S. Mukhopadhyaya<sup>5</sup> obtained many of these equations in a specialized form. In fact, A. Mukhopadhyaya,<sup>6</sup> twenty years earlier, described in some detail both the aberrancy notion of Transon and the derivation of certain special osculants. Among the papers relative to plane curve osculants the

<sup>1</sup> *Horologium Oscillatorium*, 1673.

<sup>2</sup> *Recherches sur la Courbure des Lignes et des Surfaces. Journal de Mathématiques Pures et Appliquées* (Liouville), **6**: 191-208. 1841.

<sup>3</sup> *Lezioni di Geometria Intrinseca*, Napoli, 1896.

<sup>4</sup> Some Remarks on the Historical Development and the Future Prospects of the Differential Geometry of Plane Curves. *Bulletin of the American Mathematical Society*, **22**: 317-329, 1916. (Retiring address of the Chairman of the Chicago Section of the American Mathematical Society, read at Columbus, Ohio, December 30, 1915.)

<sup>5</sup> A General Theory of Osculating Conics. *Journal of the Asiatic Society of Bengal*, New Series, vol. **4** (1908). See also: *Collected Geometric Papers*, Calcutta University Press, Calcutta, part 1, 1929, p. 103 et seq.

<sup>6</sup> On the Differential Equation of All Parabolas. *Journal of the Asiatic Society of Bengal*, vol. **57**: part II, No. iv: 316 et seq. 1888.

following may serve as helpful orientation for the reader: Enneper, A.,<sup>7</sup> Piron-dini, G.,<sup>8</sup> Lasley, J. W., Jr.,<sup>9</sup> Colucci, A.<sup>10</sup>

The present paper develops the equation of all osculating conics referred to a general reference frame. The method employed makes possible a unification of types of osculants which are seemingly quite diverse.

THE OSCULATING CONIC. Consider the equation of the conic

$$a x^2 + 2h xy + b y^2 + 2g x + 2f y + c = 0, \quad (1)$$

in which the coefficients  $a, h, b, g, f$  and  $c$  are independent of  $x$  and  $y$ .

Upon differentiation as to  $x$  and division by 2, we have

$$x a + (y + x p) h + y p b + g + p f = 0. \quad (2)$$

A second differentiation as to  $x$  yields the equation

$$a + (2p + xq) h + (p^2 + yq) b + q f = 0. \quad (3)$$

Third and fourth differentiations as to  $x$  lead us to the two equations

$$(3q + xr) h + (3pq + yr) b + rf = 0 \quad (4)$$

and

$$(4r + xs) h + (4pr + 3q^2 + ys) b + sf = 0, \quad (5)$$

where  $p, q, r$  and  $s$ , respectively, represent the first four derivatives of  $y$  as to  $x$ .

We shall suppose that (1) represents a proper conic. In that event it is possible to solve equations (4) and (5) for the ratios  $h:b:f$  and obtain

$$\begin{aligned} \rho h &= W, \\ \rho b &= V, \\ \rho f &= -9q^3 - Wx - Vy, \end{aligned} \quad . \quad (6)$$

where

$$W = 4pr^2 + 3q^2r - 3pq s,$$

$$V = 3qs - 4r^2,$$

and  $\rho$  is the proportionality factor.

Now in order for the values of  $h, b$ , and  $f$  obtained in (6) to satisfy (3) it is necessary that

$$\rho a = T - V, \quad . \quad (7)$$

where

$$T = 9q^4 - 6pq^2r + 3p^2qs - 4p^2r^2 + 3qs - 4r^2.$$

<sup>7</sup> Über die Osculatorischen Kegelschnitte ebener Curven. Zeitschrift für Mathematik und Physik, **10**: 138-153. 1874.

<sup>8</sup> Sur la Conique Osculatrice des Lignes Planes. Jornal de Ciencias e Astronomicas, **11**: 9-41. 1892.

<sup>9</sup> Penosculating Conics of a Plane Curve. Bulletin of the American Mathematical Society, **37**: 76-82. 1931.

<sup>10</sup> Sulle Coniche Osculatrici ad una Data Curva. Giornale di Mathematiche di Battaglini, **71**: 164-182. 1933.

Again, in order for the values obtained in (6) and (7) to satisfy (2) it is necessary that

$$\rho g = 9pq^3 - x(T - V) - yW. \quad (8)$$

Finally, in order for the results obtained in (6), (7) and (8) to satisfy (1) we must have

$$\rho c = 18(y - px)q^3 + x^2(T - V) + 2xyW + y^2V. \quad (9)$$

If now at any point  $(\alpha, \beta)$  of the conic (1) we compute the values of the derivatives  $p, q, r$  and  $s$  and substitute them in the expressions for  $W, V$  and  $T$  above, these quantities together with the values of  $\alpha$  and  $\beta$  will when put in (6), (7), (8) and (9) give us the coefficients in the originally chosen conic.

If, however, at any point  $(\alpha, \beta)$  of the plane we assign arbitrary values to  $p, q, r$  and  $s$  and proceed as above to compute the values of  $W, V$  and  $T$ , and with these values together with the  $\alpha$  and  $\beta$  of the originally chosen point compute the values of  $a, h, b, g, f$  and  $c$  as outlined in equations (6), (7), (8) and (9), we shall obtain the coefficients in the equation of a conic which passes through the point and takes on there the assigned values of the derivatives  $p, q, r$  and  $s$ .

Now a necessary and sufficient condition that two curves have contact of the  $k$ -th order at a point is that the two functions defining the curves together with their derivatives of the first  $k$  orders shall have the same values at the point. Consequently, the osculating conic of the curve

$$y = f(x) \quad (10)$$

at any point  $(\alpha, \beta)$  of it, has at that point the same values for the ordinate and for each of the first four derivatives  $p, q, r$  and  $s$  as does the curve (10) itself. Moreover, the conic is uniquely determined by the foregoing process. If then we form in the manner outlined the equation of the conic which has at any point  $(\alpha, \beta)$  of (10) the values of  $q, q, r$  and  $s$  provided by the curve, this conic will be the osculating conic of the curve at that point.

We have then that the osculating conic of the curve  $y = f(x)$  at any point  $(\alpha, \beta)$  of it is given by

$$a x^2 + 2h xy + b y^2 + 2g x + 2f y + c = 0.$$

Where  $a = T - V$ ,

$$h = W,$$

$$b = V,$$

$$g = 9pq^3 - \alpha(T - V) - \beta W,$$

$$f = -9q^3 - \alpha W - \beta V,$$

$$c = 18(\beta - p\alpha)q^3 + \alpha^2(T - V) + 2\alpha\beta W + \beta^2V;$$

(11)

and

$$V = 3qs - 4r^2,$$

$$W = 3q^2r - 3pq^2s + 4pr^2,$$

$$T = 9q^4 - 6pq^2r + 3p^2qs - 4p^2r^2 + 3qs - 4r^2.$$

Equation (11) may be written in the form

$$a x^2 + 2h xy + b y^2 + 2g x + 2f y + c = 0$$

where

$$a = p^2 U + L^2,$$

$$h = -pU - rL,$$

$$b = U + r^2,$$

$$g = -(p^2U + L^2)\alpha + (pU + rL)\beta + 9pq^3,$$

$$f = (pU + rL)\alpha - (U + r^2)\beta - 9q^3,$$

$$c = (p^2U + L^2)\alpha^2 - 2(pU + rL)\alpha\beta + (U + r^2)\beta^2 - 18pq^3\alpha + 18q^3\beta,$$

(12)

and

$$U = 3ps - 5r^2, L = pr - 3q^2.$$

PENOSCULATING CONICS. It will be observed that  $s$  enters equation (12) only in the combination  $3qs - 5r^2$ . If now we regard  $s$  as arbitrary and not determined by the curve (10), we have the equation of the one-parameter family of penosculating conics to  $y = f(x)$  at the point  $(\alpha, \beta)$  of it. Each and every member conic of this family has four point contact with the curve at the point  $(\alpha, \beta)$ . This is expressed analytically by means of the fact that the curve and the conic of the pencil have each the same ordinate and the same value for each of the first three derivatives  $p, q$  and  $r$  for  $x = \alpha$ . The value of  $s$  is no longer required to be that of the fourth derivative for the curve at the point, but is a free parameter whose different values distinguish the different member conics of the pencil. The equation of the pencil of conics which penosculate  $y = f(x)$  at the point  $(\alpha, \beta)$  of it is from (12) given by

$$a x^2 + 2h xy + b y^2 + 2g x + 2f y + c = 0$$

where

$$a = p^2\lambda + L^2,$$

$$h = -p\lambda - rL,$$

$$b = \lambda + r^2,$$

$$g = -p(p\alpha - \beta)\lambda - (L\alpha - r\beta)L + 9pq^3,$$

$$f = (p\alpha - \beta)\lambda + (L\alpha - r\beta)r - 9q^3,$$

$$c = (p\alpha - \beta)^2\lambda + (L\alpha - r\beta)^2 + 18q^3(\beta - p\alpha),$$

(13)

in which we have used for parameter  $\lambda = 3qs - 5r^2$ , instead of  $s$ .

THE OSCULATING PARABOLA. Among the penosculating conics given by (13), one for each value of  $\lambda$ , there is one and just one parabola, called the osculating parabola. Its equation is obtained from (13) by the particular value  $\lambda = 0$ , and is,

$$a x^2 + 2h xy + b y^2 + 2g x + 2f y + c = 0,$$

where

$$a = L^2,$$

$$h = -rL,$$

$$b = r^2,$$

$$g = -(L\alpha - r\beta)L + 9pq^3, \quad (14)$$

$$f = (L\alpha - r\beta)r - 9q^3,$$

$$c = (L\alpha - r\beta)^2 - 18q^3(p\alpha - \beta).$$

THE OSCULATING EQUILATERAL HYPERBOLA. Included in the pencil of penosculating conics (13) there is one, and only one, equilateral hyperbola, known as the osculating equilateral hyperbola. Its parameter value is  $\lambda = -(L^2 + r^2)/(1 + p^2)$ . Its equation is

$$a x^2 + 2h xy + b y^2 + 2g x + 2f y + c = 0,$$

where

$$a = 2pr - 3q^2,$$

$$h = (p^2 - 1)r - 3pq^2,$$

$$b = 3q^2 - 2pr,$$

(15)

$$g = (3q^2 - 2pr)\alpha + [(1 - p^2)r + 3pq^2]\beta - 3pq(1 + p^2),$$

$$f = [(1 - p^2)r + 3pq^2]\alpha + (2pr - 3q^2)\beta + 3q(1 + p^2),$$

$$c = (2pr - 3q^2)(\alpha^2 - \beta^2) - 2[(1 - p^2)r + 3pq^2]\alpha\beta + 6q(1 + p^2)(p\alpha - \beta).$$

SUMMARY. We have seen by means of (11) that for  $x = \alpha$  the values of the ordinate  $\beta$  and the first four derivatives  $p, q, r$  and  $s$  of the curve  $y = f(x)$  determine uniquely a 5-point conic; namely, the osculating conic of the original curve at the point  $(\alpha, \beta)$  of it.

If in equation (11) we regard  $s$  as arbitrary, we obtain the equation (13) of a 1-parameter family of 4-point conics, known as the pencil of penosculating conics. Among the conics of this pencil there is a unique 4-point parabola, known as the osculating parabola. Its equation (14) is obtained from (13) by putting  $\lambda = 3qs - 5r^2 = 0$ . There is present in this pencil a unique 4-point equilateral hyperbola, known as the osculating equilateral hyperbola. Its equation is obtained from (13) by putting  $\lambda = -\frac{L^2 + r^2}{1 + p^2}$ .

If now we regard both  $r$  and  $s$  as arbitrary, we obtain from (11) the equation of the 2-parameter family of 3-point conics. The conics of this bundle are the

totality of conics which pass through the point  $(\alpha, \beta)$  with the direction of the curve  $y = f(x)$  and have the curvature of this curve at the point. There is present in this bundle of conics a unique 1-parameter family of 3-point parabolas, known as the pencil of penosculating parabolas. Its equation may be obtained from (14) by putting  $r = \mu$ . There is present in the bundle of 3-point conics a unique pencil of 3-point equilateral hyperbolas, known as the family of penosculating equilateral hyperbolas. Its equation may be obtained from (15) by putting  $r = \mu$ . Among the conics of this bundle there is a unique 3-point circle. Its equation may be obtained from (11) by putting  $\lambda = \frac{9q^4}{1 + p^2}$ ,  $\mu = \frac{3pq^2}{1 + p^2}$ , and is given by

$$a x^2 + 2h xy + b y^2 + 2g x + 2f y + c = 0$$

where

$$a = 1,$$

$$h = 0,$$

$$b = 1,$$

$$g = -\alpha + \frac{p}{q} (1 + p^2), \quad (16)$$

$$f = -\beta - \frac{1}{q} (1 + p^2),$$

$$c = \alpha^2 + \beta^2 - \frac{2}{q} (1 + p^2)(p\alpha - \beta).$$

The center of this circle is at the point  $(x_0, y_0)$  where

$$x_0 = \alpha - \frac{p}{q} (1 + p^2),$$

$$y_0 = \beta + \frac{1}{q} (1 + p^2)$$

and the radius is given by

$$\rho = \frac{1}{q} (1 + p^2)^{3/2},$$

which identifies the circle as the circle of curvature of the given curve at the point.

If now we regard  $q$ ,  $r$  and  $s$  as arbitrary, we obtain from (11) the equation of a 3-parameter family of 2-point conics. These are the conics which pass through the point  $(\alpha, \beta)$  on  $y = f(x)$  with the same direction as the curve. There is

among the conics of this system a unique pencil of 2-point circles. The equation of this pencil may be obtained from (16) by putting  $q = v$ . Among the circles of this pencil there is one, and only one, for which the contact with the curve  $y = f(x)$  rises to 3-point contact. This is the osculating circle whose determination has been outlined above.

If further we regard  $p, q, r$  and  $s$  as arbitrary, we obtain the 4-parameter family of 1-point conics which pass throughout the point  $(\alpha, \beta)$  of the given curve.

If finally we regard  $\beta, p, q, r$  and  $s$  as arbitrary, we obtain the 5-parameter family of conics which exist in the plane.

# A PROBLEM OF ADDITIVE NUMBER THEORY AND ITS APPLICATION IN ELECTRICAL ENGINEERING

BY ALFRED BRAUER

*Department of Mathematics, University of North Carolina*

## INTRODUCTION

A short time ago, in Palmer Laboratory of Princeton University a special variable resistance was to be constructed possessing the following properties. The whole resistance was to be 30 ohms. It should have a number of fixed contact points such that each integral resistance 1, 2, 3,  $\dots$ , 30 ohms could be obtained by connecting certain two of the fixed contact points. It was of interest to find the smallest possible number of contact points.

The mathematical content of this problem is the following one. Let  $n$  be a positive integer. Find a set  $S$  of different non-negative integers  $a_1, a_2, \dots, a_k$  each less than or equal to  $n$  such that the number  $k$  of elements of  $S$  is as small as possible and such that each positive integer less than or equal to  $n$  can be represented as difference of two elements of  $S$ .

For every integer  $n$ , the number  $k = k(n)$  of such elements is a well defined number; hence  $k(n)$  is a number theoretical function. Since the  $k$  integers determine  $k(k - 1)/2$  differences, it follows that  $k(k - 1)/2 \geq n$ .

The determination of the exact value of  $k(n)$  is trivial in theory. We have only to consider the  $\binom{n+1}{k}$  sets of  $k$  non-negative integers less than or equal to  $n$  for  $k = \{\frac{1}{2} + \frac{1}{2}(8n+1)^{\frac{1}{2}}\}, \{\frac{1}{2} + \frac{1}{2}(8n+1)^{\frac{1}{2}}\} + 1$ , and so on, until we find the first set which has the required properties. But in practice, this method is completely impracticable unless we have simplifications. For instance, the problem mentioned above requires the determination of  $k(30)$ . Here we would have to consider 20,160,075 sets in order to prove that  $k(30) > 9$ .

In the following, I shall prove that  $k(30) = 10$ . I give the proof in detail although, at the first glance, this seems to be a very special result. But on one hand, my method is applicable to every given value of  $n$ . The details of the proof, however, are not exactly the same for different values of  $n$ . Therefore it is only possible to give the proof for a special value of  $n$  in order to explain the method. I use a number of lemmas for the proof of the case  $n = 30$ . For other values of  $n$ , these lemmas must be replaced by similar ones. For instance, in the case  $n = 30$  it is suitable to consider the integers  $a_i \bmod 3, 4$ , and 6; for other values of  $n$ , other moduli may be more useful.

On the other hand, we have here an example that number theoretical methods are occasionally applicable to problems in physics and in engineering. As far as I know, this paper represents the first such application of additive theory of numbers.

<sup>1</sup>  $\{z\}$  denotes the smallest integer not less than  $z$ .

Let us now consider our problem from a more general standpoint.

A set  $B$  of non-negative integers is called a basis of order  $h$  for the integer  $n$  with regard to addition if each non-negative integer less than or equal to  $n$  is the sum of  $h$  equal or different elements of  $B$ ; it is called a minimal basis of order  $h$  for  $n$  if the number  $k = k_h(n)$  of elements of  $B$  is not greater than the number of elements in any other such basis for  $n$ . The inverse function  $n = n_h(k)$  gives the greatest integer  $n$  for which a basis of order  $h$  with  $k$  elements exists. I. Schur conjectured that  $k_h(n) = O(n^{\frac{1}{h}})$ . H. Rohrbach<sup>2</sup> proved that this is true; more exactly, he proved that it is possible to construct bases for every  $n$  and every  $k$  such that

$$k_h(n) < h \cdot n^{1/h}.$$

For  $h = 2$  he obtained the sharper result

$$.4992k^2 > n_2(k) > k^2/4 + 11k/6 - 237/12.$$

Rohrbach conjectures that  $n_2(k) = k^2/4 + O(k)$ . If we consider not only the integers from 1 to  $n$ , but the set of all positive integers, then it is possible to determine bases  $B$  of order  $h$  such that the number  $k_h(m)$  of elements in  $B$  which are less than or equal to  $m$  satisfies the condition

$$k_h(m) < 2^{(h+1)/h} hm^{1/h} / e \log 2$$

for each integer  $m$ . This was proved by A. Stöhr.<sup>3</sup>

Rohrbach, in the mentioned paper, considered also more general bases  $0, a_1, a_2, \dots, a_{k-1}$  with the following property. For each integer  $m \geq 1$  and for each integer  $r$  with  $1 \leq r \leq h$  it shall be possible to choose  $h$  elements  $b_1, b_2, \dots, b_h$  of the basis such that

$$m = b_1 + b_2 + \dots + b_r - b_{r+1} - b_{r+2} - \dots - b_h.$$

In the special case  $h = 2$ , we say that such a basis is a basis with regard to addition and with regard to subtraction. Rohrbach obtains the following such basis of  $x + y$  elements for the integer  $xy + y - 1$ .

$$(1) \quad 0, 1, 2, \dots, x-1, x, 2x+1, 3x+2, \dots, yx+y-1.$$

Let  $k = x + y$  be a fixed number. Let  $n = n_2^*(k)$  be the greatest integer for which such a basis of  $k$  elements exists. It follows from (1) for  $x = [k/2]$  and  $y = k - [k/2]$  that

$$(2) \quad k(k-1)/2 \geq n_2^*(k) \geq xy + y - 1 \geq k^2/4 + k/2 - 1.$$

The problem which we considered above shows that it is of interest to consider also bases which are only bases with regard to subtraction if we are able to obtain sharper results than (2). We confine ourselves to the case that the elements

<sup>2</sup> Ein Beitrag zur additiven Zahlentheorie, Mathematische Zeitschrift, vol. 42 (1937), pp. 1-30.

<sup>3</sup> Eine Basis  $h$ -ter Ordnung für die Menge aller natürlichen Zahlen, Mathematische Zeitschrift, vol. 42 (1937), pp. 739-743.

of the basis are less than or equal to  $n$ . Here we denote the corresponding functions as above by  $n(k)$  and  $k(n)$ . Then we have  $n(k) \geq n_1^*(k)$  and  $k(n) \leq k_1^*(n)$ . From (1) we obtain a basis of 10 elements for 29, but not for 30, namely  $0, 1, 2, 3, 4, 9, 14, 19, 24, 29$  or  $0, 1, 2, 3, 4, 5, 11, 17, 23, 29$  for  $x = 4$  and  $x = 5$ , respectively.

P. Erdős noticed that instead of (1) we may choose as basis with regard to subtraction

$$(3) \quad 0, 1, 2, \dots, x-1, x, 2x+2, 3x+3, \dots, yx+y.$$

This is not a basis with regard to addition since  $2x+1$  is not obtained as a sum of two elements. It follows from (3) that

$$(4) \quad k(k-1)/2 \geq n(k) \geq k^2/4 + k/2 \quad (k \geq 5).$$

In particular, we obtain the bases  $0, 1, 2, 3, 4, 10, 15, 20, 25, 30$ , or  $0, 1, 2, 3, 4, 5, 12, 18, 24, 30$ . Hence  $k(30) \leq 10$ .

In the following, this result will be improved further. It will be shown that

$$(5) \quad k(k-1)/2 \geq n(k) \geq k^2/4 + 7k/6 - 53/12.$$

For special values of  $k$ , we obtain sharper results. It follows for instance that  $0, 1, 3, 5, 7, 9, 22, 23, 33, 34$  is such a basis. Hence  $n(10) \geq 34$ . If we compare (5) with (4), then we see that the improvement is only  $O(k)$ . But this is not surprising since it is very likely that the true value of  $n(k)$  is  $k^2/4 + O(k)$ .

### §1. Some Lemmas

We have seen that  $k(30) \leq 10$ . We want to prove that  $k(30) = 10$ . We consider all sets of 9 different integers  $a_1, a_2, \dots, a_9$  with  $0 \leq a_i \leq 30$ . For every  $S$ , let  $T$  be the set of the different positive differences  $a_\mu - a_\nu$ , and  $M$  the set of the remaining positive differences. If, for instance, three of these differences have the same value  $d$ , then  $d$  is contained once in  $T$  and twice in  $M$ . We say  $S$  is a set  $S^*$  if  $T$  is the set of all the integers from 1 to 30. We have to prove that there exists no set  $S^*$ .

$T$  contains the integer 30 if and only if  $0 \in S$  and  $30 \in S$ . Since  $30 - a_\mu - (30 - a_\nu) = a_\mu - a_\nu$ , we have

LEMMA 1. If  $[a_1, a_2, \dots, a_9]$  is a set  $S^*$ , then  $[30 - a_1, 30 - a_2, \dots, 30 - a_9]$  is such a set, too.

Since 9 numbers determine 36 positive differences, we obtain

LEMMA 2. If  $S$  is a set  $S^*$ , then  $M$  contains exactly 6 elements.

In the following, let  $d_\rho$  be the number of positive differences  $a_\mu - a_\nu$  which are divisible by  $\rho$ , and  $m_\rho$  the number of such elements of  $M$ . Denote by  $k$  and  $l$ , ( $k > l$ ), the number of elements of  $S$  in the two classes of residues mod 2. We say that  $S$  is of type  $(k, l)$  mod 2.

LEMMA 3.  $S^*$  must be of one of the types (6, 3) with  $m_2 = 3$  or (5, 4) with  $m_2 = 1$ .

*Proof.* If  $S$  is a set  $S^*$ , then  $T$  contains exactly 15 even integers and  $M$  at

most 6 even elements. Since  $z = \binom{k}{2} + \binom{l}{2}$  differences  $a_\mu - a_\nu$  are even, we have  $z \leq 15 + 6$ . This is impossible for  $k \geq 7$ . Now  $z = 18$  for  $k = 6$  and  $z = 16$  for  $k = 5$ ; hence  $m_3 = 3$  and 1, respectively.

Let us denote the number of elements of  $S$  in the 4 classes of residues mod 4 by  $k_1, k_2, l_1, l_2$  with  $k_1 + k_2 = k, l_1 + l_2 = l; k_1 \geq k_2, l_1 \geq l_2$ . We say that  $S$  is of type  $\tau = (k_1, k_2; l_1, l_2)$ .

LEMMA 4. If  $S$  is a set  $S^*$ , then only the following cases are possible: a)  $\tau = (4, 2; 3, 0), m_2 = 3, m_4 = 3$ ; b)  $\tau = (3, 3; 3, 0), m_2 = 3, m_4 = 2$ ; c)  $\tau = (4, 2; 2, 1), m_2 = 3, m_4 = 1$ ; d)  $\tau = (3, 3; 2, 1), m_2 = 3, m_4 = 0$ ; e)  $\tau = (4, 1; 2, 2), m_2 = 1, m_4 = 1$ ; f)  $\tau = (3, 2; 3, 1), m_2 = 1, m_4 = 0$ .

*Proof.*  $T$  must contain exactly 7 multiples of 4. Hence, by Lemma 3, we have  $7 \leq d_4 \leq 10$  for  $k = 6$  and  $7 \leq d_4 \leq 8$  for  $k = 5$ . On the other hand

$$(6) \quad d_4 = \binom{k_1}{2} + \binom{k_2}{2} + \binom{l_1}{2} + \binom{l_2}{2},$$

hence  $k_1 < 5$ . By Lemma 3 only the types given above and the types  $(4, 1; 3, 1)$  and  $(3, 2; 2, 2)$  are possible; but the last two must be excluded since we obtain from (6) that  $d_4 = 9$  and  $d_4 = 6$ , respectively. This contradicts  $7 \leq d_4 \leq 8$  for  $k = 5$ . In the case a, it follows from (6) that  $d_4 = 10$ ; hence  $m_4 = 3, m_2 = 3$ . In the same way the other cases are obtained.

Since  $T$  must contain 10 multiples of 3 and 5 multiples of 6, we obtain similarly

LEMMA 5. Every set  $S^*$  must be of one of the following types mod 3:  $(6, 2, 1)$  or  $(5, 4, 0)$  with  $m_3 = 6$ ;  $(5, 3, 1)$  with  $m_3 = 3$ ;  $(5, 2, 2)$  or  $(4, 4, 1)$  with  $m_3 = 2$ ;  $(4, 3, 2)$  with  $m_3 = 0$ . Hence  $m_3 \neq 1, 4$ , and 5.

LEMMA 6. Every set  $S^*$  with  $m_3 \leq 3$  must be of one of the following types mod 6:  $(4, 1; 2, 1; 1, 0)$  or  $(3, 2; 3, 0; 1, 0)$  with  $m_3 = 3, m_6 = 2$ ;  $(3, 2; 2, 1; 1, 0)$  with  $m_3 = 3, m_6 = 0$ ;  $(4, 1; 2, 0; 1, 1)$  with  $m_3 = 2, m_6 = 2$ ;  $(4, 1; 1, 1; 1, 1)$  or  $(3, 2; 2, 0; 2, 0)$ , or  $(3, 1; 3, 1; 1, 0)$  with  $m_3 = 2, m_6 = 1$ ;  $(3, 2; 2, 0; 1, 1)$  or  $(3, 1; 2, 2; 1, 0)$  with  $m_3 = 2, m_6 = 0$ , and finally  $(3, 1; 2, 1; 2, 0)$  or  $(2, 2; 3, 0; 1, 1)$  with  $m_3 = 0$ .

LEMMA 7.  $S^*$  cannot contain 5 consecutive integers or 4 consecutive integers and another pair of consecutive integers.

*Proof.* In the first case the set  $\{1, 1, 1, 2, 2, 3\}$  is contained in  $M$ . This contradicts Lemma 3. If  $a, a + 1, a + 2, a + 3$  and  $b, b + 1$  are different elements of  $S^*$ , then  $M$  contains  $\{1, 1, 1, 2, |a + 2 - b|, |a + 1 - b|, |a - b|\}$ . This is impossible by Lemma 2.

LEMMA 8. The smallest positive element  $a_8$  of every set  $S^*$  satisfies the condition  $a_8 \leq 4$ .

*Proof.* The integers  $30, 29, \dots, 30 - a_8 + 1$  are only elements of  $T$  if they are elements of  $S^*$ . Hence  $S^*$  contains  $a_8$  consecutive integers, therefore  $a_8 \leq 4$  by Lemma 7.

LEMMA 9. In every set  $S^*$  there is at least one element  $b$  such that  $10 \leq b \leq 20$ .

*Proof.* If the lemma is wrong, then 10 is not an element of  $T$ .

LEMMA 10.  $S^*$  cannot contain 6 elements greater than 19.

*Proof.* If the 6 elements are even greater than 20, then they determine 15 differences less than 10. Moreover,  $a_6 - 0 < 10$  by Lemma 8. Hence at least 16 differences are less than 10 and  $M$  contains at least 7 integers. This contradicts Lemma 2.

If, however, 20 is one of the 6 elements, then we obtain 16 differences less than 11. If  $b$  is the 9<sup>th</sup> element, then  $10 \leq b \leq 20$  by Lemma 9, and either  $20 - b$  or  $b - a_8$  is less than 11. Hence 17 differences are less than 11, and again  $M$  contains 7 elements.

LEMMA 11. If  $k$  elements of  $S$  are in the closed interval  $\{30 \cdots 30 - x\}$  and  $l$  elements in the closed interval  $\{0 \cdots y\}$  then  $S$  is not a set  $S^*$ , if

a)  $\begin{cases} k = 5, & l = 2 \\ k = 4, & l = 3 \end{cases}$  and  $\begin{cases} x \leq 6, & y \leq 2, \\ x \leq 5, & y \leq 4; \end{cases}$   
b)  $\begin{cases} k = 5, & l = 3 \\ k = 4, & l = 4 \end{cases}$  and  $\begin{cases} x \leq 9, & y \leq 3, \text{ or } x \leq 8, & y \leq 5, \\ x \leq 7, & y \leq 7. \end{cases}$

The same is valid if we permute  $x$  with  $y$  or  $k$  with  $l$ .

*Proof.* It is sufficient to prove the lemma for  $x \geq y$ . At least  $k(k-1)/2 + l(l-1)/2$  differences are less than or equal to  $x$  and at least  $kl$  differences are greater than or equal to  $30 - x - y$ . Hence the remaining differences must represent all the integers from  $x+1$  to  $30 - x - y - 1$ . This is only possible if

$$(7) \quad 36 - k(k-1)/2 - l(l-1)/2 - kl \geq 30 - 2x - y - 1.$$

If we have equality here, it is furthermore necessary that the remaining differences are different and greater than  $x$ . For  $x \leq 6, y \leq 1$  and  $x \leq 5, y \leq 3$  in case a, for  $x \leq 9, y \leq 2$ , for  $x \leq 8, y \leq 4$ , and  $x \leq 7, y \leq 6$  in case b, the condition (7) is not satisfied. In the remaining cases both hands in (7) are equal. If  $a'_1$  is the last element of  $S$  in the case b, then  $30 - x > a'_1 > y$  by Lemma 9, and it is necessary that

$$(8) \quad 30 - x - y = (30 - x - a'_1) + (a'_1 - y) \geq x + 1 + x + 2 = 2x + 3.$$

But (8) is not satisfied in the remaining cases of b.

In the case a, let  $a'_1, a'_2$  be the last elements of  $S$ . Since the case b is already proved, it is sufficient to assume that  $30 - x > a'_1 > a'_2 > y$ . It is necessary now that

$$(9) \quad \begin{aligned} 30 - x - y &= (30 - x - a'_1) + (a'_1 - a'_2) + (a'_2 - y) \\ &\geq x + 1 + x + 2 + x + 3 = 3x + 6. \end{aligned}$$

This does not hold if  $x = 6, y = 2$ . For  $x = 5, y = 4$  it is only satisfied if the 3 differences in (9) are 6, 7, and 8 in any order; then  $a'_1 \geq 17, a'_2 \leq 12$ . If  $a'_1 \geq 18$ , then the differences of  $k$  of the given elements and  $a'_1$ , of  $a'_2$  and  $l$  such elements, and  $a'_1 - a'_2$  are less than 13. Hence 8 differences  $d$ , satisfy the condition  $6 \leq d, \leq 12$ . If  $a'_1 = 17$ , then  $a'_1 - 4 = 13$ , and 9 of the  $d$ , satisfy  $6 \leq d, \leq 13$ . In both cases at least 2 of these  $d$ , are equal.

LEMMA 12. Lemma 11 b holds also for  $x = 8, y = 6$  and  $x = 6, y = 8$ .

*Proof.* By Lemma 11, we may assume that 22 and 6  $\in S$ . The 28 differences of the given 8 elements represent the 23 integers 1, 2,  $\dots$ , 8 and 16, 17,  $\dots$ , 30. Hence  $M$  contains more than 6 elements if we obtain 2 further such representations using  $a'_1$ . But either  $22 - a'_1 \leq 8$  or  $a'_1 - 6 \leq 8$  by Lemma 9 and, for  $a'_1 \neq 15$ , either  $30 - a'_1 \geq 16$  or  $a'_1 - 0 \geq 16$ . For  $a'_1 = 15$  we obtain 2 representations of 15, hence 31 representations of 24 numbers. This is impossible.

LEMMA 13. Let  $a_1 > a_2 > \dots > a_8$  be the elements of  $S$  and  $g_1 > g_2 > \dots$  those of these elements which belong to one of the residue classes mod 2, and by  $h_1 > h_2 > \dots$  those of the other class. Denote by  $\sigma_1, \sigma_2, \mu_1, \mu_2$  the sums of the 36 differences, of the even differences, of all the elements of  $M$ , and of the even such elements, respectively. If  $S$  is a set  $S^*$ , then

$$(10) \quad \begin{aligned} \sigma &= 8a_1 + 6a_2 + 4a_3 + 2a_4 - 2a_6 - 4a_7 - 6a_8 = 465 + \mu, \\ &= 5g_1 + 3g_2 + g_3 - g_4 - 3g_5 - 5g_6 + 2h_1 - 2h_3 \\ &\quad = 240 + \mu_2 \text{ if } S \text{ is of type (6, 3),} \\ (11) \quad \sigma_2 &= 4g_1 + 2g_2 - 2g_4 - 4g_5 + 3h_1 + h_2 - h_3 - 3h_4 \\ &\quad = 240 + \mu_2 \text{ if } S \text{ is of type (5, 4).} \end{aligned}$$

*Proof.*  $a_8 = 0; 1 + 2 + \dots + 30 = 465; 2 + 4 + \dots + 30 = 240$ .

Denote by  $[a_*, a_\lambda, \dots, a_r]$  the totality of sets  $S$  containing  $a_*, a_\lambda, \dots, a_r$ . If these elements determine the elements  $b_1, b_2, \dots, b_r$  of  $M$ , then we write  $\{b_1, b_2, \dots, b_r\}$ . If a further element of  $S$  is given, it may determine further elements  $c_1, c_2, \dots, c_r$ , then we write  $\{\{c_1, c_2, \dots, c_r\}\}$ , and so on.

## §2: The case $a_8 = 4$

Our result could be proved in 2 lines by saying: Consider each of the possible 20160075 systems  $S$ ; for each given system it is easy to show that it is not a set  $S^*$ . But then the whole work must be done really by the reader. We want to give the proof in a form that it can be checked in a short time. It cannot be avoided that we have to consider a number of cases. We try to reduce this number as much as possible. The proofs of the single cases are different although we apply certain common principles.

In the following, we assume that  $S$  is a set  $S^*$ . Therefore  $M$  contains exactly 6 elements by Lemma 2. It follows from Lemma 8 that  $a_8 \leq 4$ . If  $a_8 = 4$ , then 29, 28, and 27  $\in S$ , hence  $[30, 29, 28, 27, 4, 0], \{1, 1, 2\}$ . Let  $a'_1$  be one of the remaining elements of  $S$ , then it is impossible by Lemma 11a for  $k = 5, l = 2, x = 5, y = 4$  that  $a'_1 \geq 25$ . In the same way it follows for  $k = 4, l = 3, x = 3, y = 5$  that  $a'_1 \leq 5$  is impossible. Hence 22  $\in T$  only if  $S$  contains at least one of the integers 8, 7, 6, or 22.

- I.  $6 \in S$ , then  $\{\{2, 23, 24\}\}$ . This is impossible by Lemma 5 since  $m_8 = 1$ .
- II.  $7 \in S, [30, 29, 28, 27, 7, 4, 0], \{3, 23\}, 23 > a'_1 > 9$  by Lemma 11b. Hence 19  $\in T$  only if 11, 10, or 19  $\in S$ . If 11 or 10  $\in S$ , then 4 or 20  $\in M$ . Both contradicts Lemma 3. If 19  $\in S$ , then 18  $\in T$  only if 12, 22, or 18  $\in S$  since 11  $\in S$  was impossible. But then 7 or 1  $\in M$ . This contradicts Lemma 5.

III.  $8 \in S$ ,  $[30, 29, 28, 27, 8, 4, 0], \{1, 1, 2, 4\}, 24 > a'_s > 9$  by Lemma 11 and 12. Since  $m_2 > 2$ ,  $m_4 \geq 1$ , and  $29 \not\equiv 27 \pmod{4}$ , it follows from Lemma 4 that  $S$  is of type  $(4, 2; 2, 1)$ . Hence  $a'_s \not\equiv 0 \pmod{4}$  and  $18 \in T$  only if  $11, 10, 22$ , or  $18 \in S$ . In the first 3 cases  $\{\{3, 19\}\}, \{\{2, 20\}\}, \{\{8, 22\}\}$ , respectively. This contradicts Lemma 3. If  $18 \in S$ , then  $16 \in T$  only if the last element of  $S$  is 13 or 11. But then 6 is not contained in  $T$ .

IV.  $22 \in S$ ,  $[30, 29, 28, 27, 22, 4, 0], a'_s < 20$  by Lemma 10. Hence, by I, II, and III,  $21 \in T$  only if  $9 \in S$ . But then  $\{1, 1, 2, 5, 18\}$ . It follows from Lemma 3 and 5 that  $b_6 \equiv 0 \pmod{6}$ , hence  $m_8 = m_6 = 2$ , and  $S$  is of type  $(4, 1; 2, 0; 1, 1)$  by Lemma 6. This is here impossible.

### §3. The case $a_8 = 3$

We have  $[30, 29, 28, 3, 0]$ . Here  $24 \in T$  only if  $6, 5, 4, 27$ , or  $24 \in S$ .

I.  $27 \in S$ ,  $[30, 29, 28, 27, 3, 0], \{1, 1, 2, 3, 27\}, 25 > a'_s > 5$ . Hence  $23 \in T$  only if  $7, 6$ , or  $23 \in S$ . If  $23 \in S$ , then  $20 > a'_s > 7$  by Lemma 10 and 11. Hence  $22 \in T$  only if  $8 \in S$ ; but then  $\{\{5, 20\}\}$ . This is impossible. If  $6 \in S$ , then  $\{\{3, 24\}\}$ . If  $7 \in S$ , then  $23 > a'_s > 9$ , and  $a'_s \not\equiv 3 \pmod{4}$  by Lemma 4f. Hence  $19 \in T$  only if  $10$  or  $22 \in S$ . But then  $\{\{3, 7\}\}$  and  $\{\{7, 22\}\}$ .

II.  $4 \in S$ ,  $[30, 29, 28, 4, 3, 0], \{1, 1, 25, 26\}, 25 > a'_s > 6$ . Hence  $23 \in T$  only if  $7$  or  $23 \in S$ . If  $7 \in S$ , then  $\{\{3, 4\}\}$ . If  $23 \in S$ , then  $22 > a'_s > 7$ . Hence  $22 \in T$  only if  $8 \in S$ ; but then  $\{\{4, 5\}\}$ . Both cases are impossible by Lemma 3.

III.  $5 \in S$ ,  $[30, 29, 28, 5, 3, 0], \{1, 2, 25\}, 26 > a'_s > 6$ . Hence  $22 \in T$  only if  $8, 7, 25$ , or  $22 \in S$ . If  $25 \in S$ , then  $\{\{3, 5, 25\}\}$ ; this is impossible by Lemma 5. If  $7 \in S$ , then  $\{\{2, 23\}\}, 23 > a'_s > 9$ ; it follows from Lemma 3 that  $a_4 \equiv a_5 \equiv 1 \pmod{2}$ , hence  $20$  is not contained in  $T$ . If  $8 \in S$ , then  $\{\{3, 5\}\}, 25 > a'_s > 9$ . By Lemma 5,  $S$  must be of type  $(4, 4, 1)$ , hence  $a'_s \not\equiv 1 \pmod{3}$ . Now  $19 \in T$  only if  $11$  or  $24 \in S$ , but then  $8$  or  $24 \in M$ ; this contradicts Lemma 3. If  $22 \in S$ , then  $22 > a'_s$ . Since the cases  $7$  or  $8 \in S$  are already proved,  $21 \in T$  only if  $9$  or  $21 \in S$ ; then  $S$  is of type  $(5, 4) \pmod{2}$ . This contradicts Lemma 3 since  $\{\{6\}\}$  and  $\{\{8\}\}$ .

IV.  $6 \in S$ ,  $[30, 29, 28, 6, 3, 0], \{1, 3\}$ . By I, III, and II,  $21 \in T$  only if  $9, 8, 7, 24$ , or  $21 \in S$ .

IVa.  $9 \in S$ ,  $[30, 29, 28, 9, 6, 3, 0], \{\{3, 6\}\}, 6 > m_8 \geq 3$ . It follows from Lemma 5 that  $m_8 = 3$ , hence  $m_6 = 1$ . This is impossible by Lemma 6.

IVb.  $8 \in S$ ,  $[30, 29, 28, 8, 6, 3, 0], \{\{2, 22\}\}, 24 > a'_s > 9$ . Hence  $19 \in T$  only if  $11, 10, 22$ , or  $19 \in S$ . In the first 3 cases  $\{\{\{3, 5\}\}\}, \{\{\{2, 20\}\}\}$ , and  $\{\{\{6, 22\}\}\}$ . This contradicts Lemma 3. If  $19 \in S$ , then  $\{\{\{11\}\}\}$ , and  $a'_s$ , even. Hence  $18 \in T$  only if  $12$  or  $18 \in S$ , but then  $9$  or  $1 \in M$ .

IVc.  $7 \in S$ ,  $[30, 29, 28, 7, 6, 3, 0], \{\{1, 22, 23\}\}, 23 > a'_s > 9$ . Hence  $20 \in T$  only if  $10$  or  $20 \in S$ . If  $10 \in S$ , then  $\{\{\{3, 7\}\}\}$ . If  $20 \in S$ , then  $b_6 \equiv 3 \pmod{6}$  by Lemma 3 and 5, hence  $m_8 = 2, m_6 = 0$ . This contradicts Lemma 6 since  $S$  contains elements of each of the 6 classes of residues mod 6.

IVd.  $24 \in S$ ,  $[30, 29, 28, 24, 6, 3, 0], \{\{6, 24\}\}$ . Since  $m_8 = 3$ , it follows from Lemma 5 that  $S$  is of type  $(5, 3, 1)$ , hence  $a_6 \equiv a_8 \equiv 29$  or  $a_6 \equiv a_8 \equiv 28 \pmod{3}$ . These 3 congruent numbers must have the 3 remaining multiples of 3 as differences, namely 9, 12, and 15. This is impossible since  $9 + 12 \not\equiv 15$ .

IVe.  $21 \in S$ , [30, 29, 28, 21, 6, 3, 0]. By IVa, b, c, d, and III,  $20 \in T$  only if 10, 26, 23, or  $20 \in S$ . In the last 3 cases  $\{\{2, 3, 23, 26\}\}$ ,  $\{\{2, 6, 7, 23\}\}$ , and  $\{\{1, 8, 9\}\}$ . This is impossible by Lemma 3 and 4e. If  $10 \in S$ , then  $\{\{7, 18\}\}$ . Here  $a'_s \not\equiv 0 \pmod{3}$  by Lemma 5. Hence, by II, III, IVb and since the cases 23 or  $20 \in S$  are already proved,  $17 \in T$  only if 13, 11, or  $17 \in S$ . But then  $T$  does not contain 12, 12, 16, respectively.

V.  $24 \in S$ , [30, 29, 28, 24, 3, 0]. Here  $23 \in T$  only if 7, 26, or  $23 \in S$  by IV, III, and II. If  $26 \in S$ , then  $\{2, 2, 4, 26\}$ . This is impossible by Lemma 3. If  $23 \in S$ , then  $\{1, 1, 5, 6\}$ ,  $21 > a'_s > 7$ . Hence  $22 \in T$  only if  $8 \in S$ . Here  $\{\{5, 20\}\}$ ; this contradicts Lemma 3. If  $7 \in S$ , then  $\{1, 4, 21\}$ ,  $23 > a'_s > 8$ . Hence  $20 \in T$  only if 10, 9, or  $20 \in S$ . By Lemma 4e and 6, the cases 10 or 9  $\in S$  are impossible since  $\{\{3, 7\}\}$  and  $\{\{2, 6, 21\}\}$ . If  $20 \in S$ , then  $\{\{4, 17\}\}$ ,  $20 > a'_s > 8$ . Here  $\sigma = 528 - 2a_6$ ,  $\mu = 47 + b_6$ , and by (10)  $2a_6 + b_6 = 16$ . This is impossible since  $a_6 > 8$ .

#### §4. The case $a_6 = 2$

We have [30, 29, 2, 0],  $26 \in T$  only if 4, 3, 28, or  $26 \in S$ .

I.  $28 \in S$ . By §3,  $25 \in T$  only if 5, 4, 27, or  $25 \in S$ .

Ia.  $27$  or  $4 \in S$ . Here  $\{1, 1, 2, 2, 27, 28\}$ , and  $\{2, 2, 26, 28\}$ . This is impossible by Lemma 5 and 3.

Ib.  $25 \in S$ , [30, 29, 28, 25, 2, 0],  $\{1, 2, 28\}$ ,  $24 > a'_s > 4$ . Hence  $24 \in T$  only if 6 or  $5 \in S$ . If  $5 \in S$ , then  $\{\{3, 5, 23, 25\}\}$ . If  $6 \in S$ , then  $\{\{4, 23\}\}$ ,  $23 > a'_s > 8$ , and by (10)  $2a_6 + b_6 = 17$ . This is impossible.

Ic.  $5 \in S$ , [30, 29, 28, 5, 2, 0],  $\{1, 2, 28\}$ ,  $26 > a'_s > 6$ . Hence  $22 \in T$  only if 8, 7, 24, or  $22 \in S$ .

Ica.  $7 \in S$ ,  $\{\{2, 5, 23\}\}$ ,  $23 > a'_s > 9$ . But  $a_4 = 23$  by (10). This is impossible.

Icb.  $24 \in S$ , [30, 29, 28, 24, 5, 2, 0],  $\{\{5, 24\}\}$ ,  $22 > a'_s > 8$ . Hence  $21 \in T$  only if 9 or  $21 \in S$ . Then  $\{\{4, 19\}\}$ , and  $\{\{3, 19\}\}$ .

Icy.  $8 \in S$ , [30, 29, 28, 8, 5, 2, 0],  $\{\{3\}\}$ ,  $24 > a'_s > 9$ . Now  $19 \in T$  only if 11, 10, 21, or  $19 \in S$ . If 10 or 11  $\in S$ , then  $\{\{2, 8\}\}$  and  $\{\{3, 6\}\}$ ; this contradicts Lemma 3 and 6. If  $21 \in S$ , then  $\{\{8, 21\}\}$ ,  $20 \geq a'_s \geq 10$ . Hence  $\sigma_2 - g_2 = 268$ ,  $\mu_2 = 38$ , hence  $a_6 = g_2 = 10$  by (11). But then  $20 \in M$ . This is impossible. If finally  $19 \in S$ , then  $\{\{11\}\}$ , hence  $b_6 \equiv 0 \pmod{6}$ ,  $m_3 = 2$ ,  $m_6 = 1$ . This is here impossible by Lemma 6.

Icd.  $22 \in S$ , [30, 29, 28, 22, 5, 2, 0],  $22 > a'_s > 6$ . By Icy and  $\alpha$ ,  $21 \in T$  only if 9 or  $21 \in S$ , moreover  $18 \in T$  only if 12, 11, 10, 20, or  $18 \in S$ , and  $14 \in T$  only if 16, 15, 14, or  $19 \in S$ . This gives a contradiction since only 2 elements of  $S$  are unknown.

II.  $3 \in S$ , [30, 29, 3, 2, 0]. By §3,  $25 \in T$  only if 5, 4, 27, or  $25 \in S$ .

IIa.  $4 \in S$ , [30, 29, 4, 3, 2, 0],  $25 > a'_s > 6$ . Hence  $24 \in T$  only if  $24 \in S$ . But then  $23 > a'_s > 7$ , and 23 is not in  $T$ .

IIb.  $5 \in S$ , [30, 29, 5, 3, 2, 0],  $\{1, 2, 3, 27\}$ ,  $26 > a'_s > 6$ . Hence  $23 \in T$  only if 7, 25, or  $23 \in S$ . If  $7 \in S$ , then  $\{\{2, 5\}\}$ ; this is impossible by Lemma 3. If  $25 \in S$ , then  $\{\{5, 25\}\}$ ,  $22 > a'_s > 8$ . But  $a_4 = 24$  by (10); this is impossible. If  $23 \in S$ , then  $22 > a'_s > 7$ , hence  $22 \in T$  only if  $8 \in S$ ; then  $\{\{3, 5, 6\}\}$ .

IIc.  $27 \in S$ ,  $[30, 29, 27, 3, 2, 0]$ ,  $\{1, 2, 3, 27, 27\}$ ,  $25 > a'_s > 5$ . Hence  $23 \in T$  only if 7, 6, or  $23 \in S$ . If  $6 \in S$ , then  $\{\{3, 24\}\}$ . If  $23 \in S$ , then  $21 > a'_s > 7$ , hence  $22 \in T$  only if  $8 \in S$ ; but then  $\{\{6, 21\}\}$ . If  $7 \in S$ , then  $23 > a'_s > 9$ , and  $21 \in T$  only if  $21 \in S$ . Here  $a'_s \equiv 2 \pmod{12}$  by Lemma 4f and 5, hence  $a'_s = 14$ , but then  $\{\{7, 14\}\}$ .

IId.  $25 \in S$ ,  $[30, 29, 25, 3, 2, 0]$ ,  $25 > a'_s$ . By IIb,  $24 \in T$  only if 6 or  $24 \in S$ . If  $6 \in S$ , then  $\{1, 3, 4, 23, 27\}$ ,  $22 > a'_s > 8$ , and  $a'_s \not\equiv 1 \pmod{4}$  by Lemma 4e. Hence 21 is not contained in  $T$ . If  $24 \in S$ , then  $\{1, 1, 5, 22, 27\}$ ,  $21 > a'_s > 8$ ,  $m_3 = 2$ ,  $m_6 = 0$ , hence  $a'_s \not\equiv 4 \pmod{6}$ . Now  $20 \in T$  only if 9 or  $20 \in S$ . But then  $\{\{6, 21\}\}$  and  $\{\{4, 5\}\}$ .

III.  $26 \in S$ ,  $[30, 29, 26, 2, 0]$ . Here  $25 \in T$  only if 5, 4, 27, or  $25 \in S$ .

IIIa.  $27 \in S$ ,  $[30, 29, 27, 26, 2, 0]$ ,  $\{1, 2, 3, 27\}$ ,  $24 > a'_s > 5$ . Now  $23 \in T$  only if 7, 6, or  $23 \in S$ . If  $23$  or  $6 \in S$ , then  $\{\{3, 4\}\}$  and  $\{\{4, 24\}\}$ . This is impossible by Lemma 3 and 4b. If  $7 \in S$ , then  $23 > a'_s > 8$ . Now  $21 \in T$  only if 9 or  $21 \in S$ . But then  $\{\{2, 7\}\}$  and  $\{\{5, 19\}\}$ . Both cases are impossible, the latter since  $\sigma = 534 - 2a_6 \leq 516$  and  $\mu = 57$ . This contradicts (10).

IIIb.  $25 \in S$ . Here  $24 > a'_s > 4$  and  $22 \in T$  only if 8, 7 or  $22 \in S$ .

IIIb $\alpha$ .  $7 \in S$ ,  $[30, 29, 26, 25, 7, 2, 0]$ ,  $\{1, 4, 5, 23\}$ ,  $23 > a'_s > 8$ . Hence  $21 \in T$  only if 9 or  $21 \in S$ . This is impossible since  $\{\{2, 7\}\}$  or  $\{\{4, 5\}\}$ .

IIIb $\beta$ .  $8 \in S$ ,  $[30, 29, 26, 25, 8, 2, 0]$ ,  $\{1, 4\}$ ,  $25 > a'_s > 8$ . Here  $20 \in T$  only if 10, 9, 22, or  $20 \in S$ . If  $9 \in S$ , then  $\{\{1, 17, 21\}\}$ ,  $\sigma - \mu = 462 - b_6$ . This contradicts (10). If  $10 \in S$ , then  $\{\{2, 8\}\}$ . The last element  $h_3$  must be odd, but  $h_3 = 14$  by (11).  $22 \in S$  is impossible since  $\{\{3, 4, 8, 22\}\}$ . If  $20 \in S$ , then  $\{\{5, 6, 18\}\}$ ; this contradicts Lemma 4c.

IIIb $\gamma$ .  $22 \in S$ ,  $[30, 29, 26, 25, 22, 2, 0]$ ,  $\{1, 3, 4, 4\}$ ,  $21 > a'_s > 6$ . By IIIb $\beta$ ,  $21 \in S$  only if  $9 \in S$ . This contradicts Lemma 5 since  $\{\{7, 20\}\}$ .

IIIc.  $4 \in S$ ,  $[30, 29, 26, 4, 2, 0]$ ,  $\{2, 4, 26\}$ ,  $25 > a'_s > 5$ . Here  $g_3 = 2h_3 - 4$  by (11). Hence  $S$  must contain one of the pairs 7, 10; 9, 14; 11, 18; 13, 22; But then  $\{\{22\}\}$ ,  $\{\{12\}\}$ ,  $\{\{18\}\}$ ,  $\{\{4\}\}$ .

IIId.  $5 \in S$ ,  $[30, 29, 26, 5, 2, 0]$ ,  $\{3, 24\}$ ,  $26 > a'_s > 5$ . By IIIb,  $23 \in T$  only if 7, 6, or  $23 \in S$ . If  $6 \in S$ , then  $22 > a'_s > 8$ , hence 22 is not contained in  $T$ . If  $7 \in S$ , then  $\{\{2, 5\}\}$ ,  $23 > a'_s > 8$ ; moreover  $a'_s$  even by Lemma 3. Hence  $17 \in T$  only if 12 or  $22 \in S$ . But here  $\{\{5, 7\}\}$  and  $\{\{4, 22\}\}$ . If  $23 \in S$ , then  $\{\{3, 21\}\}$ ,  $22 > a'_s > 7$ . Now  $22 \in T$  only if  $8 \in S$ , but then  $\{\{3, 6\}\}$ .

IV.  $4 \in S$ ,  $[30, 29, 4, 2, 0]$ . By I, II, and III,  $24 \in T$  only if 6, 5, or  $24 \in S$ .

IVa.  $5 \in S$ ,  $\{1, 2, 25\}$ ,  $26 > a'_s > 6$ . Now  $23 \in T$  only if 7, 25, or  $23 \in S$ . If 7 or  $25 \in S$ , then  $\{\{2, 3, 5\}\}$  and  $\{\{4, 5, 25\}\}$ . If  $23 \in S$ , then  $22 > a'_s > 7$ . Now  $22 \in T$  only if  $8 \in S$ , but then  $\{\{3, 4, 21\}\}$ .

IVb.  $6 \in S$ ,  $[30, 29, 6, 4, 2, 0]$ ,  $\{2, 2, 4\}$ ,  $a'_s > 6$ . Now  $3g_2 - 2h_3 = 44$  by (11), hence either 22 and 11, or 26 and 17  $\in S$ . But then  $\{\{18\}\}$  or  $\{\{4\}\}$ .

IVc.  $24 \in S$ ,  $[30, 29, 24, 4, 2, 0]$ ,  $23 \in T$  only if 7, 27, 25, or  $23 \in S$  by III, IVa, IVb.

IVc $\alpha$ .  $27 \in S$ ,  $\{2, 2, 3, 25, 27\}$ ,  $22 > a'_s > 8$ . Hence  $21 \in T$  only if 9 or  $21 \in S$ , but then  $\{\{5, 20\}\}$  and  $\{\{3, 6\}\}$ .

IVc $\beta$ .  $25 \in S$ ,  $\{1, 2, 4, 5, 25\}$ ,  $22 > a'_s > 8$ .  $S$  is of type  $(2, 2; 3, 0; 1, 1)$  by Lemma 6, hence  $a'_s \not\equiv 0 \pmod{3}$ . Therefore  $19 \in T$  only if 11, 10 or  $19 \in S$ .

If 10 or 19  $\in S$ , then  $\{\{6, 20\}\}$  and  $\{\{5, 6\}\}$ . If 11  $\in S$ , then  $b_6 = 10$  by (10), hence  $g_8 = 8$  by (11). This is impossible.

IVcγ.  $23 \in S$ ,  $\{1, 2, 6\}$ ,  $22 > a'_s > 7$ . Hence  $18 \in T$  only if 12, 11, 20, or  $18 \in S$ . If  $20 \in S$ , then  $\{\{4, 20\}\}$ . If  $18 \in S$ , then  $\{\{5, 6\}\}$ ,  $h_8 = 17$  by (11), and  $\{\{6\}\}$ . If  $11 \in S$ , then  $\{\{7, 19\}\}$ . Hence  $b_6 = 0 \pmod{6}$  and  $S$  must be of type  $(4, 1; 2, 0; 1, 1)$ . If  $12 \in S$ , then  $\{\{12\}\}$ , and  $h_8 = 11$ . Each of these cases is impossible.

IVcδ.  $7 \in S$ ,  $\{2, 5, 22\}$ ,  $23 > a'_s > 8$ . Here  $21 \in T$  only if 9 or  $21 \in S$ . Hence  $\{\{2, 20\}\}$ , or  $\{\{3, 17\}\}$ . The latter case is impossible since  $\sigma - \mu = 461 - b_6$ .

### §5. The case $a_8 = 1$

We have here only to consider the case  $a_2 = 29$  since  $[30, a_2, \dots, 0]$  corresponds to  $[30, \dots, 30 - a_2, 0]$  by Lemma 1 and is therefore already treated in the case  $a_2 \neq 29$ . If we have  $[30, 29, 1, 0]$ , then  $27 \in T$  only if 3, 28, or  $27 \in S$  by §4. Since  $[30, 29, 28, 1, 0]$  corresponds to  $[30, 29, 2, 1, 0]$  it is proved by §4. The cases  $27 \in S$  and  $3 \in S$  correspond to each other, hence only one of them must be considered. If  $S = [30, 29, 27, 1, 0]$ , then  $25 \in T$  only if 5, 4, 26, or  $25 \in S$  by §3 and 4.

I.  $26 \in S$ ,  $[30, 29, 27, 26, 1, 0]$ ,  $\{1, 1, 3, 26, 29\}$ ,  $24 > a'_s > 5$ . Hence  $24 \in T$  only if  $6 \in S$ , but then  $23 > a'_s > 8$ . Here  $22 \in T$  only if  $22 \in S$ , then  $\{\{4\}\}$ .

II.  $25 \in S$ ,  $[30, 29, 27, 25, 1, 0]$ ,  $\{1, 2, 29\}$ ,  $24 > a'_s > 4$ . Now  $23 \in T$  only if 7, 6, or  $23 \in S$ . If  $7 \in S$ , then  $23 > a'_s > 8$ , and  $21 \in T$  only if 9, 22, or  $21 \in S$ . But then  $\{\{2, 18, 20\}\}$ ,  $\{\{3, 5, 22\}\}$ , and  $\{\{4, 6, 20\}\}$ . If  $6 \in S$ , then  $\{\{5, 24\}\}$ ,  $23 > a'_s > 8$ . Hence  $22 \in T$  only if  $22 \in S$ , but then  $3 \in M$ . If  $23 \in S$ , then  $\{\{2, 4\}\}$ ,  $20 > a'_s > 7$ . By Lemma 6,  $21 \in T$  only if  $8 \in S$ ; but then  $\{\{22\}\}$ .

III.  $4 \in S$ ,  $[30, 29, 27, 4, 1, 0]$ ,  $\{1, 3, 26, 29\}$ ,  $25 > a'_s > 5$ . Here  $24 \in T$  only if 6 or  $24 \in S$ . If  $6 \in S$ , then  $\{\{2, 23\}\}$ . If  $24 \in S$ , then  $22 > a'_s > 8$ ; hence  $22$  is not in  $T$ .

IV.  $5 \in S$ ,  $[30, 29, 27, 5, 1, 0]$ ,  $\{1, 29\}$ ,  $26 > a'_s > 5$ . Here  $23 \in T$  only if 7, 6, 24, or  $23 \in S$ .

IVa.  $6 \in S$ ,  $\{\{1, 5, 24\}\}$ . By Lemma 4e and 13,  $h_8 = 24$ , hence  $\{\{24\}\}$ .

IVb.  $7 \in S$ ,  $\{\{2, 22\}\}$ ,  $23 > a'_s > 9$ . Here  $21 \in T$  only if  $22$  or  $21 \in S$ . But then  $\{\{5, 7\}\}$  and  $\{\{6, 20\}\}$ .

IVc.  $24 \in S$ ,  $\{\{3, 5, 24\}\}$ ,  $22 > a'_s > 8$ , but  $h_8 = 6$  by Lemma 4e and 13.

IVd.  $23 \in S$ ,  $\{\{4, 22\}\}$ ,  $22 > a'_s > 7$ . Here  $21 \in T$  only if 9, 8, or  $21 \in S$ , but then  $\{\{4, 18\}\}$ ,  $\{\{3, 7\}\}$ , and  $\{\{2, 6\}\}$ .

### §6. Bases with regard to subtraction

**THEOREM 1.** Let  $x$  and  $y$  be positive integers, and  $k = 3x + y$ . The integers

$$0, 1, 2, \dots, x - 1,$$

$$2x - 1, 3x - 1, \dots, (y + 1)x - 1,$$

$$(2y + 4)x - 2, (2y + 4)x - 1, \dots, (2y + 5)x - 3,$$

$$(3y + 6)x - 3, (3y + 6)x - 2, \dots, (3y + 7)x - 4$$

form a basis of  $k$  elements with regard to subtraction for the integer  $n = (3y + 7)x - 4$ .

*Proof.* We have to prove that every integer  $t$  with  $0 < t \leq (3y + 7)x - 4$  can be obtained as difference of 2 numbers of the system (13). Set

$$t = qx + r, \quad 0 \leq r \leq x - 1.$$

1. If  $q = 0$ , then  $t = r - 0$  is the difference of 2 elements of (13).

2. If  $1 \leq q \leq y$ , then we write  $t$  in the form

$$t = qx + r = \{(q + 1)x - 1\} - \{x - 1 - r\}.$$

The first term is an element of the second line, the second an element of the first line of (13).

3.  $q = y + 1, 0 \leq r \leq x - 1$ . Here we obtain  $t$  as difference of the first element of the fourth line and an element of the third line,

$$t = (y + 1)x + r = \{(3y + 6)x - 3\} - \{(2y + 4)x - 2 + (x - 1 - r)\}.$$

4.  $q = y + 2, 0 \leq r \leq x - 2$ . Here  $t$  is the difference of the last element of the fourth row and one of the first  $x - 1$  elements of the third line,

$$t = \{(3y + 7)x - 4\} - \{(2y + 4)x - 2 + (x - 2 - r)\}.$$

5.  $y + 3 \leq q \leq 2y + 2, r = -1, 0, 1, \dots, x - 2$ . We have

$$t = \{(2y + 4)x - 2 + (r + 1)\} - \{(2y + 4 - q)x - 1\}.$$

The first term is an element of the third row and the last term an element of the second row since  $0 \leq r + 1 \leq x - 1$  and  $2 \leq 2y + 4 - q \leq y + 1$ .

6.  $q = 2y + 3, r = -1, 0, 1, \dots, x - 2$ . Here we obtain

$$t = \{(2y + 4)x - 2\} - \{x - 2 - r\},$$

$$(0 \leq x - 2 - r \leq x - 1).$$

7.  $q = 2y + 4, r = -1, 0, 1, \dots, x - 3$ . We have

$$t = \{(2y + 5)x - 3\} - \{x - 3 - r\},$$

$$(0 \leq x - 3 - r \leq x - 2).$$

8.  $2y + 5 \leq q \leq 3y + 4, r = -2, -1, 0, \dots, x - 3$ . Here

$$t = \{(3y + 6)x - 3 + (r + 2)\} - \{(3y + 6 - q)x - 1\},$$

$$(2 \leq 3y + 6 - q \leq y + 1).$$

9.  $q = 3y + 5, r = -2, -1, 0, \dots, x - 3$ . We obtain

$$t = \{(3y + 6)x - 3\} - \{x - 3 - r\}.$$

10.  $q = 3y + 6, r = -2, -1, 0, \dots, x - 4$ . Here

$$t = \{(3y + 7)x - 4\} - \{x - 4 - r\}.$$

In order to prove that the basis (13) is better than the basis (1) of Rohrbach we prove

**THEOREM 2.** For every integer  $k$  there exists a basis with regard to subtraction for an integer  $n$  such that

$$n \geq k^2/4 + 7k/6 - 53/12.$$

*Proof.* We have nothing to prove for  $k = 1, 2, 3$ . Assume  $k \geq 4$ . We choose  $x = \left[ \frac{k+5}{6} \right]$ ,  $y = k - 3 \left[ \frac{k+5}{6} \right]$ , and set  $\left[ \frac{k+5}{6} \right] = \left[ \frac{k+5}{6} \right] + \delta$ , then  $y \geq 1$ . It follows from (13) that

$$\begin{aligned} n &= (3y + 7)x - 4 = \{3k - 3(k+5)/2 + 9\delta + 7\}\{(k+5)/6 - \delta\} - 4 \\ &= k^2/4 + 7k/6 - 53/12 + 8\delta - 9\delta^2 \geq k^2/4 + 7k/6 - 53/12 \end{aligned}$$

since  $\delta \leq 5/6$ .

**COROLLARY.** We have  $n(k) \geq k^2/4 + 7k/6 - 53/12 + \gamma$  where  $\gamma = 0, 13/12, 5/3, 7/4, 4/3, 5/12$  for  $k = 1, 2, 3, 4, 5, 0 \pmod{6}$ , respectively.

## ON WAVES AND PARTICLES

BY NATHAN ROSEN

*Department of Physics, University of North Carolina*

The development of the quantum theory has brought about important changes in many of our physical concepts. Perhaps one of the most striking changes has taken place in the description of the motion of a particle. In classical physics we described the motion of a particle by giving the coordinates of the particle, in some specified reference system, as functions of the time. The motion was considered to be governed by Newton's laws, and the equations of motion, in the form of ordinary differential equations, were to be solved for the coordinates as functions of the time, subject to certain initial conditions specifying the coordinates and velocity components at a given instant of time. The initial values of the coordinates and velocity components or momenta completely determined the motion of the particle, and these initial values could be measured, at least in principle, to any desired degree of accuracy.

In quantum mechanics, on the other hand, the motion of a particle is described by means of a wave function. With this function one can calculate the probability of finding the particle in the neighborhood of a point with given coordinates, or the probability that the particle will have a given velocity. The wave function is obtained as the solution of the Schroedinger equation, which is a partial differential equation involving the coordinates and time as independent variables. Through the use of the wave function one can explain, among other things, certain diffraction phenomena that have been observed in connection with electrons and that could not be explained on the basis of the particle description provided by classical physics. In part, the difference in approach in the two theories is associated with the fact that, while in classical physics one can know the initial values of the coordinates and momenta as accurately as one likes, in quantum mechanics the Heisenberg uncertainty principle provides a limit to the accuracy to which one can measure simultaneously a coordinate and the corresponding momentum, so that one cannot know the initial values sufficiently well to determine completely the subsequent motion and hence must resort to probabilities.

In recent years there has been a good deal of discussion among physicists concerning the interpretation of wave mechanics and the description of nature provided by the latter.<sup>1</sup> It is important to have an understanding of the extent to which classical concepts can be carried over into the quantum theory and the conditions under which they conflict with the formalism of this theory. The purpose of the present paper is to investigate some aspects of these questions.

<sup>1</sup> E.g., Einstein, Podolsky and Rosen, Phys. Rev. **47**: 777 (1935); N. Bohr, Phys. Rev. **48**: 696 (1935).

1. Let us begin with the time-dependent Schrödinger equation for a single particle of mass  $m$ , moving in a field of force described by the potential energy function  $V(x, y, z)$ ,

$$\frac{ih}{2\pi} \frac{\partial \psi}{\partial t} = -\frac{h^2}{8\pi^2 m} \nabla^2 \psi + V\psi. \quad (1)$$

Let us now carry out a procedure that has been used repeatedly in the literature.<sup>2</sup> We write

$$\psi = Ae^{i\pi W/h}, \quad (2)$$

where  $A$  and  $W$  are real functions. Substituting this into (1) and breaking up the equation into real and imaginary parts, we get

$$-A \frac{\partial W}{\partial t} = -\frac{h^2}{8\pi^2 m} \nabla^2 A + \frac{1}{2m} A(\nabla W)^2 + VA, \quad (3)$$

$$\frac{h}{2\pi} \frac{\partial A}{\partial t} = -\frac{h}{4\pi m} (A \nabla^2 W + 2\nabla A \cdot \nabla W). \quad (4)$$

Eq. (3), after division by  $A$ , becomes

$$-\frac{\partial W}{\partial t} = \frac{1}{2m} (\nabla W)^2 + V - \frac{h^2}{8\pi^2 m} \frac{\nabla^2 A}{A}, \quad (5)$$

and eq. (4), after multiplication by  $4\pi A/h$ , can be written

$$\frac{\partial}{\partial t} (A^2) = -\frac{1}{m} \nabla \cdot (A^2 \nabla W). \quad (6)$$

In order to interpret these equations, let us first consider the limiting case of classical mechanics obtained by allowing Planck's constant  $h$  to go to zero. It is customary to say that in this limit the last term on the right of (5) vanishes and can be discarded. Actually, this may or may not be the case—we shall return to a consideration of this point—but let us assume for the present that it is. Then (5) becomes

$$-\frac{\partial W}{\partial t} = \frac{1}{2m} (\nabla W)^2 + V. \quad (7)$$

For this limiting case, both sides of (4) vanish, so that (6) is no longer a consequence, although there appears to be no objections to retaining it.

Equation (7) is the well-known Hamilton partial differential equation of classical mechanics. Since  $V$  does not depend on the time, one can take

$$W = S(x, y, z) - Et \quad (8)$$

where  $E$  is a constant, the energy of the particle, and (7) becomes

<sup>2</sup> L. Brillouin, J. de Physique **7**: 353 (1926); G. Wentzel, Zeits. f. Physik **38**: 518 (1926); E. Madelung, Zeits. f. Physik **40**: 322 (1926); E. H. Kennard, Phys. Rev. **31**: 876 (1928).

$$\frac{1}{2m} (\nabla S)^2 + V = E, \quad (9)$$

the Hamilton-Jacobi equation.

A solution for  $S$  having been found, the momentum  $\mathbf{p}$  of the particle at any point is given by

$$\mathbf{p} = \nabla W = \nabla S, \quad (10)$$

so that the path of the particle is an orthogonal trajectory of the family of surfaces  $S = \text{constant}$ . To every solution  $S$  there thus corresponds an infinite number ( $\infty^2$ ) of possible paths. The particular path followed by the given particle and its position along this path at a certain instant of time depend on the initial conditions of the problem.

If a solution  $S$  is given without any initial conditions, then the whole infinity of paths and of positions must be associated with the solution. One can give it a statistical interpretation by supposing that  $S$  describes the behavior of an aggregate (Gibbs ensemble) of non-interacting particles, each of which is executing a possible motion of our given particle. From this point of view, eq. (6) takes on a definite significance. If we denote by  $\rho$  the density of the particles of the ensemble and set

$$\rho = A^2 \quad (11)$$

then, since it follows from (10) that the velocity at any point is

$$\mathbf{v} = -\nabla W, \quad (12)$$

(6) has the form of the equation of continuity,

$$\frac{\partial \rho}{\partial t} = -\nabla \cdot (\rho \mathbf{v}), \quad (13)$$

expressing conservation of the number of particles.

2. Let us now consider the situation in quantum mechanics where we no longer have  $\hbar = 0$ . The only essential difference between this case and the previous one is in the presence on the right-hand side of equation (5) of the term

$$V_q = -\frac{\hbar^2}{8\pi^2 m} \frac{\nabla^2 A}{A}. \quad (14)$$

It is natural to try to make use of the same procedure as above. Obviously this can be done provided one replaces  $V$  everywhere by

$$V' = V + V_q. \quad (15)$$

Thus, we now have eq. (5) instead of (7), and, accepting eq. (8) we have, instead of (9),

$$\frac{1}{2m} (\nabla S)^2 + V - \frac{\hbar^2}{8\pi^2 m} \frac{\nabla^2 A}{A} = E. \quad (16)$$

Equations (10), (11), and (12) continue to hold, and (13) is now a necessary consequence of (4).

This suggests using the same interpretation as before:

The possible paths of the particle are the normal trajectories to the surfaces  $S = \text{constant}$ . The actual path and the position of the particle along this path will be determined by the initial conditions. If a solution for  $S$  and  $A$  is given without any initial conditions, then the whole aggregate of paths and positions must be associated with it, that is, the solution can be thought of as describing the behavior of an ensemble of particles, each of which is executing a possible motion of the particle under consideration.

However, the picture now differs in one important respect from that of the classical case. We saw above that in the classical case the particles of the ensemble were non-interacting: the motion of each one was determined by the given force field and was independent of the other particles. This is no longer true in the quantum-mechanical case. The motion of each particle is now determined by the "potential energy function"  $V'$  which, in addition to the given potential energy  $V$ , contains the term  $V_a$ .<sup>3</sup> The latter is given by (14), or if we make use of (11), by the relation

$$V_a = \frac{\hbar^2}{32\pi^2 m} \left[ \left( \frac{\nabla \rho}{\rho} \right)^2 - 2 \frac{\nabla^2 \rho}{\rho} \right]. \quad (17)$$

This means that the motion of each particle of the ensemble depends on the density with which all the members of the ensemble are distributed, so that effectively we have an interaction among the particles. Nevertheless, from the standpoint considered here, the various members of the ensemble are to be regarded as *possible* representatives of the particle under consideration, only one of them being the *actual* representative in a particular case. In this respect the present point of view is different from that of the "hydrodynamical interpretation" of the wave function proposed by Madelung.<sup>2</sup>

Let us now suppose that we have a particle the behavior of which is described by the Schroedinger equation (1) and which is known to be in a certain state described by a particular wave function  $\psi$  satisfying (1). (According to the principles of the quantum theory such a knowledge of the state of the particle can be obtained by carrying out a suitable series of measurements.<sup>4</sup>) From the wave function  $\psi$  and its complex conjugate  $\psi^*$  one gets

$$\rho = \psi \psi^*, \quad W = \frac{\hbar}{4\pi i} \ln \frac{\psi}{\psi^*}, \quad (18)$$

and hence

$$\mathbf{p} = \frac{\hbar}{4\pi i} \left( \frac{\nabla \psi}{\psi} - \frac{\nabla \psi^*}{\psi^*} \right), \quad \mathbf{v} = \frac{\hbar}{4\pi i m} \left( \frac{\nabla \psi}{\psi} - \frac{\nabla \psi^*}{\psi^*} \right). \quad (19)$$

However, as was pointed out above, the wave function describes many possible kinds of motion of the particle. To get the actual motion, one needs additional

<sup>2</sup> Kennard, 1 c.

<sup>4</sup> P. A. M. Dirac, *The Principles of Quantum Mechanics*, Oxford, 1935.

information. For example, if  $\rho$  differs from zero over a finite part of space, we do not know where the particle is located at a given instant of time and, to find out about this, must carry out a measurement of its position. Once we have determined the position of the particle, then from (19) we can calculate its momentum and velocity. To be sure, the momentum and velocity, as calculated in this way, are the values the particle had just before the measurement and not after it, for the measurement of the position changes the momentum (and hence the velocity) in an unpredictable way, as Heisenberg showed.<sup>6</sup> The information that we get about the particle in this way is complete, but, unfortunately, refers only to the past. The trouble is that, once the measurement has been carried out, the state of the particle has been altered and is no longer described by the same wave function  $\psi$ .

Obviously, it is not possible to confirm the results obtained by repeating the measurement. Moreover, there does not seem to be anything practical that one can do with the information. However, it is interesting that one can maintain a self-consistent picture of the particle, as a particle with a definite position and velocity, within the framework of the quantum theory.

From the present point of view the picture is then as follows: If we have a particle which is classically in a potential energy field  $V$ , it will be described wave-mechanically by some wave function  $\psi$  corresponding to a certain state. In that case its motion, from the standpoint of classical mechanics, will depend, not on  $V$ , but on  $V'$ , as given by (15). The quantum-mechanical correction  $V_q$  can be interpreted as a kind of interaction among the various particles of a statistical ensemble. However, such an interpretation should not be taken too seriously, since in an actual case only *one* particle is present.

3. The preceding discussion has been based on the assumption that one can go continuously from quantum mechanics to classical mechanics by letting  $h$  go to zero. The possibility of such a continuous transition suggests a continuity of interpretation, and this was the idea made use of above. However, there are cases in which a continuous transition is not possible, that is, in which  $V_q$  does not tend to zero as  $h$  goes to zero. For example, for a free particle of mass  $m$ , moving along a straight line, which we take as the  $X$ -axis of coordinates, we can have a wave function of the form

$$\psi = \sqrt{2} \cos \left( \frac{2\pi px}{h} \right) e^{-\frac{2\pi i Et}{h}}, \quad (20)$$

where  $p$  is the magnitude of the momentum,  $E$  is the energy, and the density has been normalized to unity per unit distance. A simple calculation shows that in this case

$$V_q = \frac{p^2}{2m}, \quad (21)$$

which is independent of  $h$  and hence remains finite as  $h$  tends to zero.

A wave function for which  $V_q$  does not go to zero with  $h$  describes a state which

<sup>6</sup> W. Heisenberg, Zeits. f. Physik, 43: 172 (1927).

does not have any direct classical analogue. However, by means of the superposition principle, such a state can be described by a combination of two or more wave functions. In the example just considered one can write

$$\psi = \psi_1 + \psi_2, \quad (22)$$

where

$$\psi_1 = \frac{1}{\sqrt{2}} e^{\frac{2\pi i}{\hbar} (px - Et)}, \quad \psi_2 = \frac{1}{\sqrt{2}} e^{-\frac{2\pi i}{\hbar} (px + Et)}. \quad (23)$$

One finds in this particular example that for each of the wave functions  $\psi_1$  and  $\psi_2$  separately the corresponding  $V_q$  turns out to vanish whatever the value of  $\hbar$ , so that these functions describe states which go over continuously into classical states. In some cases the wave functions required for the linear combination may possess singularities.

For convenience, let us call a wave function for which  $V_q$  tends to zero with  $\hbar$ , a simple wave function, and the state described by it, a simple state, while, if  $V_q$  does not go to zero as  $\hbar$  goes to zero, let us call the wave function and state composite. Physically, a simple wave function is one, the amplitude of which does not change very rapidly with distance as  $\hbar$  becomes small, while for a composite function the amplitude has a "ripple" the wave length of which becomes shorter and shorter as  $\hbar$  approaches zero. It appears then that the considerations of the two preceding sections are to be applied to simple wave functions, and not to composite ones.

That a composite wave function may lead to difficulties if one attempts to apply the interpretation discussed above can be seen from the following example:

Suppose we have a wave function  $\psi$  which can be written in the form

$$\psi = \phi(x, y, z) e^{-\frac{2\pi Et}{\hbar}}, \quad (24)$$

where  $\phi$  is real. It is evident that in this case  $A = \phi$ ,  $W = -Et$  and  $S = 0$ . From (10) or (19) it follows then that  $p = v = 0$ , which means that the particle is completely at rest. Such a conclusion is hard to accept. In the Schrödinger theory, wave functions of the form (24) describe many of the stationary states of the hydrogen atom, and the idea that in each of these the electron is at rest seems to be in contradiction with our concepts of angular momentum and polarization and their relation to the selection rules for the emission of radiation.

One can try to modify the interpretation in the case of a composite wave function. Let us write the composite function as a linear combination of simple functions (properly normalized) with constant coefficients:

$$\psi = c_1 \psi_1 + c_2 \psi_2 + \dots \quad (25)$$

Each of the simple functions can be interpreted in terms of the motion of a particle (or aggregate of particles) in the corresponding potential-energy field  $V'$ . It is natural to interpret the composite function  $\psi$  in (25) as describing an aggregate of particles executing motions corresponding to each of the simple

functions in the linear combination, the fraction of the aggregate having a type of motion corresponding to  $\psi_k$  being proportional to  $|c_k|^2$ . However, if we accept this point of view, then we neglect in  $\psi\psi^*$  the various cross-product terms like  $\psi_1\psi_2^*$ , etc. This means that we give up the possibility of *interference* of two wave functions, and interference is, of course, an essential feature of wave mechanics.

Instead of referring to simple and composite wave functions, one can discuss the matter in terms of standing and running waves. As long as we are dealing with wave functions representing running waves, i.e., waves having a definite direction of propagation everywhere, we can interpret such wave functions by means of the motion of particles. If, however, we are dealing with standing waves or, more generally, with waves which are superpositions of two or more waves having different directions of propagation, then an interpretation based on moving particles appears possible only if one gives up interference phenomena. We have here apparently a manifestation of Bohr's complementarity principle: particles and interference are mutually exclusive.

#### SUMMARY

From the Schroedinger equation one can obtain an equation which has the form of the Hamilton-Jacobi equation of classical physics but with an extra term, a quantum-mechanical correction, present. The solutions of the Schroedinger equation are of two kinds: (a) those for which the quantum-mechanical correction goes to zero as  $h$  goes to zero, and (b) those for which it does not. Solutions of the first kind can be interpreted in terms of particles executing classical motion in a field of force modified by the correction. For solutions of the second kind, such an interpretation leads to the conclusion that in many of the stationary atomic states the particles are motionless, or else one must abandon the possibility of describing interference phenomena.

# PENICILLIN: V. MYCOLOGICAL ASPECTS OF PENICILLIN PRODUCTION

By KENNETH B. RAPER<sup>1</sup> AND DOROTHY F. ALEXANDER<sup>2</sup>

*Fermentation Division, Northern Regional Research Laboratory,<sup>3</sup> Peoria, Illinois*

WITH FOURTEEN TEXT FIGURES

## INTRODUCTION

During the past four years penicillin and *Penicillium notatum* Westling, the mold at first employed in its production, have occupied the attention of microbiologists and chemists alike to a greater extent than any other fungus or fungus product. The story of the discovery of penicillin by Prof. Alexander Fleming in 1928 at St. Mary's Hospital in London (11, 15) has been told and retold, and it is now familiar to scientists and laymen alike. It is also well known that for a decade after Fleming's discovery little study was given to penicillin. During that period almost nothing was known of its vast potentialities except the observations by the discoverer (11) and by Raistrick and coworkers (6) that it effectively prevented the growth of many, but not all, disease-producing bacteria. While Fleming was not unmindful of the possible value of penicillin as a therapeutic agent, his primary interest in this antibiotic substance was as an aid in the isolation of pathogenic bacteria (11).

Beginning in 1940 with the first published work of Florey and associates (3), a renewed interest in penicillin developed. Since the publication a year later by this Oxford group of a second paper embodying the results of actual clinical trials, penicillin has received intense and ever-increasing study by investigators, at first in England, subsequently in the United States, and now all over the world.

Much has been written regarding the clinical use of penicillin by the Floreys and their associates (3, 17, 18), Pulvertaft (36), and others in England, and by Herrell (24, 25), Dawson and associates (9, 27), Mahoney *et al.* (33), Lyons (31), Keefer *et al.* (29), and others in this country. The mode of action of penicillin has been considered by Fleming (13), Hobby *et al.* (26, 27), Rammelkamp (37), and others. Extensive research has been devoted to the development and improvement of methods of assaying penicillin, and much work on this subject has been published, including reports by Abraham *et al.* (1), Fleming (14), Foster (19, 20, 21), Schmidt and Moyer (42), McMahan (32), Joslyn (28), and others. While much information concerning the production of penicillin has been classified as "restricted information" because of its bearing upon the war effort, a number of papers describing early methods of production were published

<sup>1</sup>B.A., University of North Carolina, 1929; Assistant in Botany, *Ibid.*, 1926-1929; M.A., George Washington Univ., 1931; M.A., Harvard, 1935, Ph.D., *Ibid.*, 1936. Senior Microbiologist, Fermentation Division, Northern Regional Research Laboratory, Peoria, Ill.

<sup>2</sup>Junior Microbiologist, *Ibid.*

<sup>3</sup>One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

(1, 5, 22). More recently other reports have appeared dealing in general with production methods (7, 10) but withholding information regarding specific nutrient combinations currently employed to secure high yields. Papers supplying such information are now in press (39) or in preparation (34, 35), however, and will appear during the next few months.

During this same four-year period when so much has been reported concerning other aspects of the penicillin development (a comprehensive bibliography, as of June 1944, contained more than 300 references), very little has been published regarding the molds which produce penicillin, and almost nothing has been written about the cultures that are largely responsible for the current production of this important drug. For this reason, certain mycological aspects of penicillin production will be considered in the present paper. Particular attention will be given to results obtained during a two-year study of natural variation and penicillin production in four different members of the *Penicillium notatum-chrysogenum* group which are now generally used for the commercial production of penicillin. This number includes (1) *Penicillium notatum* Westling, the original Fleming isolate, and strains developed from it, including NRRL 1249.B21, widely used for the production of penicillin in surface culture; (2) *P. notatum*, NRRL 832, generally employed for the production of penicillin in submerged culture; (3) *P. notatum*, NRRL 1950, and strains derived from it that produce high yields in surface culture; and (4) *P. chrysogenum* Thom, NRRL 1951, and strains derived therefrom, including NRRL 1951.B25, used for the production of penicillin in surface and submerged culture.

#### METHODS

In the present investigations, cultural and microscopic observations have been made upon colonies growing in petri-dish culture upon the following media:

(1) Czapek's solution agar

NaNO <sub>3</sub>	3 0	g.
K <sub>2</sub> HPO <sub>4</sub>	1 0	"
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0 5	"
KCl	0 5	"
FeSO <sub>4</sub> 7H <sub>2</sub> O	0 01	"
Sucrose	30.0	"
Agar	15 0	"
Distilled water	1 0	liter
pH approximately 7 0, not adjusted.		

(2) "E" medium (enriched Czapek's solution agar)

Formula as above, except that 2% agar and 1% concentrated corn steeping liquor are added.  
pH adjusted to 7 0 prior to the addition of agar.

(3) Malt extract agar

Malt extract (Difco)	20.0	g.
Dextrose	20.0	"
Peptone	1.0	"
Agar	25.0	"
Distilled water	1.0	liter
pH approximately 4 7; not adjusted.		

Of these media, Czapek's is the most widely used for the cultivation of the *Penicillia* and is generally the most diagnostic. "E" medium has proved especially useful in our investigation of variants of *Penicillium chrysogenum*, NRRL 1951. Malt agar enables most forms to sporulate abundantly but produces colonies that are generally of little diagnostic value.

Two types of cultures have been employed: (1) Plates inoculated with single colonies centrally placed, and (2) plates inoculated at three points equidistant from each other and from the margin of the plate. Cultures of the first type are particularly valuable in revealing relative rates of growth, basic colony patterns, etc. Cultures of the second type are more useful for microscopic study since they sporulate earlier and in marginal areas produce fruiting structures which can be examined directly with the low-power objectives of a compound microscope. Cultural and microscopic observations have generally been made from 10 days to 2 weeks after inoculation. Critical cultural observations include rate of growth, colony texture, abundance of sporulation, color of conidial areas, and exudate formation. Microscopic examinations should include observations of the penicilli *in situ* under comparatively low powers, followed by examination of the same structures under oil immersion to establish details of morphology and to obtain measurements of parts. Comparative data have been assembled from cultures grown either at 24°C. or at room temperatures of 23°-26°C.

Subcultures investigated have been obtained by a variety of means including: (1) Isolation from colonies in dilution plates, (2) isolation from variant sectors of single colonies, (3) isolation from colonies in streak plates inoculated from production flasks, and (4) isolation of single conidia from selected stocks. Dilution cultures were made by preparing a spore suspension and employing the usual bacteriological techniques, and were most productive of variant types when made from cultures grown upon sporulation or other nutrient-rich media. Sector isolates were made from colony areas differing markedly from the main colony under observation. A colony of NRRL 1249.B21 (used for the production of penicillin in surface culture) showing such sectors is illustrated in Figure 1, A. Streak plates inoculated with 1-week-old mycelia taken from submerged production cultures have proved especially useful in our studies of variation in NRRL 1951. A typical plate of this type, 1-week-old, made from a substrain of NRRL 1951.B25, is shown in Figure 1, B. The isolation of variants from cultures of this kind is easily accomplished. Single spore isolations were made in the following manner: A dilute spore suspension was prepared and spread over the surface of marked areas of an agar plate. The plate was then incubated overnight, and the spores allowed to germinate. Single spores were located with the aid of a low-power binocular, and their positions marked. Each was subsequently checked with an 8 mm. objective to insure that there were no other spores in the adjacent area. With a platinum micro scalpel, a minute block of agar bearing the selected spore was removed and transplanted to a separate agar plate.

The following nutrient solution, developed at the Northern Regional Research

Laboratory (34, 39), was used in testing strains for their ability to produce penicillin in surface culture:

Lactose	40 000 g
MgSO <sub>4</sub> 7H <sub>2</sub> O	0 250 "
KH <sub>2</sub> PO <sub>4</sub>	0 500 "
NaNO <sub>3</sub>	3 000 "
Zn (as ZnSO <sub>4</sub> )	0 010 "
Concentrated corn steeping liquor*	90 0 ml
or	
Dried steep liquor solids†	50 0 g
Distilled water to make	1.0 liter

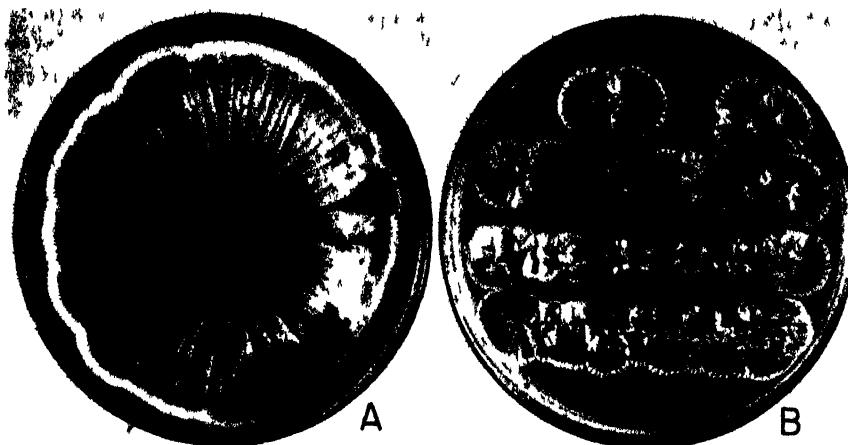


FIG. 1. Cultures from which variant substrains could be isolated. A 3 week old colony of NRRL 1249 B21 on I medium showing conspicuous sectors, B streak culture seeded with mycelia (in pellet form) from a 1 week old submerged production culture of *P. chrysogenum* NRRL 1951 B25.

During the early part of the investigation, 50 cc of this solution was dispensed in 200-cc Erlenmeyer flasks. Later 75 cc was used in 300-cc flasks. The depth of the solution in both cases was approximately three-fourths inch. Cultures were run in duplicate, or triplicate, and in most instances each flask was assayed daily from the fourth through the eighth days. Dry spores produced in cultures grown upon sporulation media of the type recommended by Moyer and Coghill (34) were used as inoculum. Sufficient spores were added to each flask to form a thin but uniform covering film. In cases where the sporulation medium did not provide adequate spores, flasks were inoculated with small amounts of 7 to 10-day-old bran cultures. Incubation was at 24°C.

\* Concentrated corn steeping liquor normally contains approximately 55% solids. Adjustments in the amount used should be made if liquors containing more or less solids are employed.

† A spray dried powder marketed by the American Maize Products Co. Roby Indiana, under the trade name "Amaizo steep water solids."

The following nutrient solution, developed at the Northern Regional Research Laboratory (35, 39), was employed in testing strains for their capacity to produce penicillin in submerged culture:

Lactose	20.000 g.
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.250 "
KH <sub>2</sub> PO <sub>4</sub>	0.500 "
NaNO <sub>3</sub>	3.000 "
Zn (as ZnSO <sub>4</sub> )	0.010 "
Concentrated corn steeping liquor	40.000 ml
or	
Dried steep-liquor solids	25.000 g.
Distilled water to make	1.000 liter

Sterile CaCO<sub>3</sub>, at the rate of approximately 1 g./100 cc. of culture solution, was added just prior to inoculation. Shakers of two types were employed, and cultures of different sizes were used. In early tests cultures were grown in 300 cc. Erlenmeyer flasks containing 125 cc. of nutrient solution and were incubated on two Ross-Kershaw shakers adjusted to run at as nearly the same speed as possible (210 r.p.m.). In later tests cultures were grown in 1-liter Erlenmeyer flasks containing 175 cc. of culture solution and were incubated on a single rocker shaker patterned after a type developed at the University of Wisconsin. No antifoam agent was employed. In comparative tests approximately equal yields were obtained on the two types of shakers, but the single rocker shaker was found to be the more satisfactory. It was of larger capacity and was designed so that every flask received exactly the same agitation. Inoculations were made with spore suspensions washed from 5- to 7-day-old cultures grown upon sporulation media. Spore suspensions were fairly dense—usually of the order of 50-60 million per cc. Inoculum was usually added at the rate of 0.25 to 0.50 cc. of such a suspension per 100 cc. of culture solution. Reasonable variations in the amount of inoculum appeared to have little influence upon the amount of penicillin produced. Cultures were incubated at 24°C. Each flask was assayed daily from the third through the sixth or seventh day, by which time the yields were falling off rapidly.

All assays were made by the cylinder-plate method and were performed by William H. Schmidt and assistants. The reader is referred to the paper by Schmidt and Moyer (42) for details of this method.

#### THE FLEMING STRAIN AND NRRL 1249 B21

"While working with *staphylococcus* variants a number of culture-plates were set aside on the laboratory bench and examined from time to time. In the examinations these plates were necessarily exposed to the air and they became contaminated with various micro-organisms. It was noticed that around a large colony of a contaminating mould the *staphylococcus* colonies became transparent and were obviously undergoing lysis."

In this manner Professor Fleming in May 1929 (11) announced the discovery of the penicillin-producing mold which was subsequently to be employed in all of the early investigations on penicillin (1, 3, 6, 11), and from which strains were to be developed that would contribute substantially to the commercial production

of this drug. That this mold had been isolated in pure culture, correctly identified, and maintained in culture collections in England and in the United States during the period from 1929 to 1940 proved to be most important to the early clinical evaluation (1, 3) and to the resultant interest in the manufacture of penicillin (7).

In his original paper Fleming (11) very briefly described the appearance and behavior of this mold and concluded: "In its morphology this organism is a *Penicillium*, and in all its characteristics it most closely resembles *P. rubrum*." Shortly thereafter Fleming gave the culture to Professor Harold Raistrick of the London School of Hygiene and Tropical Medicine. He, in turn, sent it to Dr. Charles Thom, Principal Mycologist of the U. S. Department of Agriculture, with the request that he verify or correct the identification. After careful study, Dr. Thom concluded that, while it did not completely fit Westling's description of *P. notatum* or duplicate his type, which had been received eight years before, the strain was closer to this species than to any other. Since that time, this name has been consistently applied whenever reference to the culture has been made.

During the period from 1930 to 1940, when the senior author was associated with Dr. Thom, the Fleming culture (Thom's No. 5112.1) was maintained continuously upon slants of Czapek's solution agar. Throughout this period the strain possessed the consistent and rather distinctive appearance which still characterizes it. When cultures from the Thom Collection were brought to the Northern Regional Research Laboratory in 1940, this strain was assigned the serial number 824 in the permanent culture collection of the Laboratory. It is our considered opinion that culture 824 represents with reasonable exactness the mold as it was studied originally by Fleming (11) and by Raistrick and associates (6).

Professor Florey and associates (1, 3) used a culture which they obtained directly from Professor Fleming, and when he and Dr. Heatley came to the Northern Regional Research Laboratory in July 1941, they brought this culture with them. To it was assigned the designation NRRL 1209. In our work it is commonly referred to as the "Heatley strain." It was employed by Drs. Moyer and Heatley in the early investigations conducted at this Laboratory from July to December 1941. Culturally and microscopically it duplicates strain 824 in all essential characteristics.

Early in January 1942 a culture was received from Dr. George A. Harrop of the Squibb Institute for Medical Research which was found to be better for penicillin production than Nos. 824, 1209, and other versions of the Fleming strain then in our collection (34). It was designated as NRRL 1249 and was later sent out to investigators under this number. The culture had been received by the Squibb Institute in October 1940 from the Thom Collection as No. 5112.1, and therefore was of the same immediate origin as our No. 824 referred to above. At the Squibb Institute, strain selections had been made, and penicillin yields increased materially.

Culture 1249 was studied exhaustively at the Northern Regional Research Laboratory during the succeeding months, and it was through the use of this

strain that Moyer and Coghill (34) were able to solve many important nutritional problems leading to the commercial production of penicillin by the surface method.

While NRRL 1249, or the Squibb strain, was the most satisfactory of any then in our possession, it was not stable in production cultures, and difficulties were encountered in securing uniform results. As reported by Moyer and Coghill (34), areas of white, flocculent, non-sporulating mycelium of varying extent frequently developed in sporulation tubes and flasks, and also in production cultures. This white growth, when isolated and tested, produced approximately half as much penicillin as did the normal, sporulating culture. At the same time, there were indications that variation within this strain was not entirely in the direction of lower penicillin yields. Culture selections were made and tested, and from the best of these cultures, designated No. 1249.B, two successive series of monospore cultures were isolated. Penicillin production in surface culture by these isolates was tested by Moyer and Coghill (34), and a number of high-yielding monosporous strains were discovered. Of these, No. 21 appeared to be the most satisfactory. Culture NRRL 1249.B21, thus derived, was intensively studied after this time (December 1942). It was sent out to many certified industries and research groups in the United States and Allied Nations and was adopted almost exclusively as the strain for commercial production of penicillin in surface culture. Only within the past few months have other high-yielding strains, subsequently developed, begun to supplant it (see pp. 94, 102, 103).

Strain NRRL 1249.B21 is unstable in culture. When cultivated upon sporulation or other nutrient-rich media, variant types regularly develop, and when the strain is recultivated upon such substrata through a few successive generations, the variants commonly come to dominate the culture, or even completely to supplant the parent type. In our experience these variants are regularly characterized by reduced penicillin production, and no substrains of 1249.B21 have been isolated which produce consistently higher yields of penicillin than the parent strain.

Comparisons have been made of the cultural and microscopic characteristics of the Fleming strain, NRRL 824, and the numerous substrains derived from it. In this way, it has been possible to correlate high penicillin production with certain recognizable cultural and morphological characteristics; strains lacking such characteristics regularly produce lower yields. This relationship can be presented by considering the outstanding mycological characteristics of certain selected strains and comparing their penicillin production.

#### NRRL 824—The Fleming Strain.

Colonies upon Czapek's solution agar in 3-point inoculation, 3.5 to 4.0 cm. in diameter after 10 days at 24°C., showing conspicuous radial furrows, velvety, heavy-sporing (Figure 2, A), sage to artemisia green or even lily green (Plate XLVII),<sup>4</sup> becoming slate olive (Plate XLVII) in age, producing abundant yellow to amber exudate in small droplets; colony

<sup>4</sup> Citations of specific colors refer to plates in Ridgway's "Color Standards and Color Nomenclature," 1912, Washington, D. C.

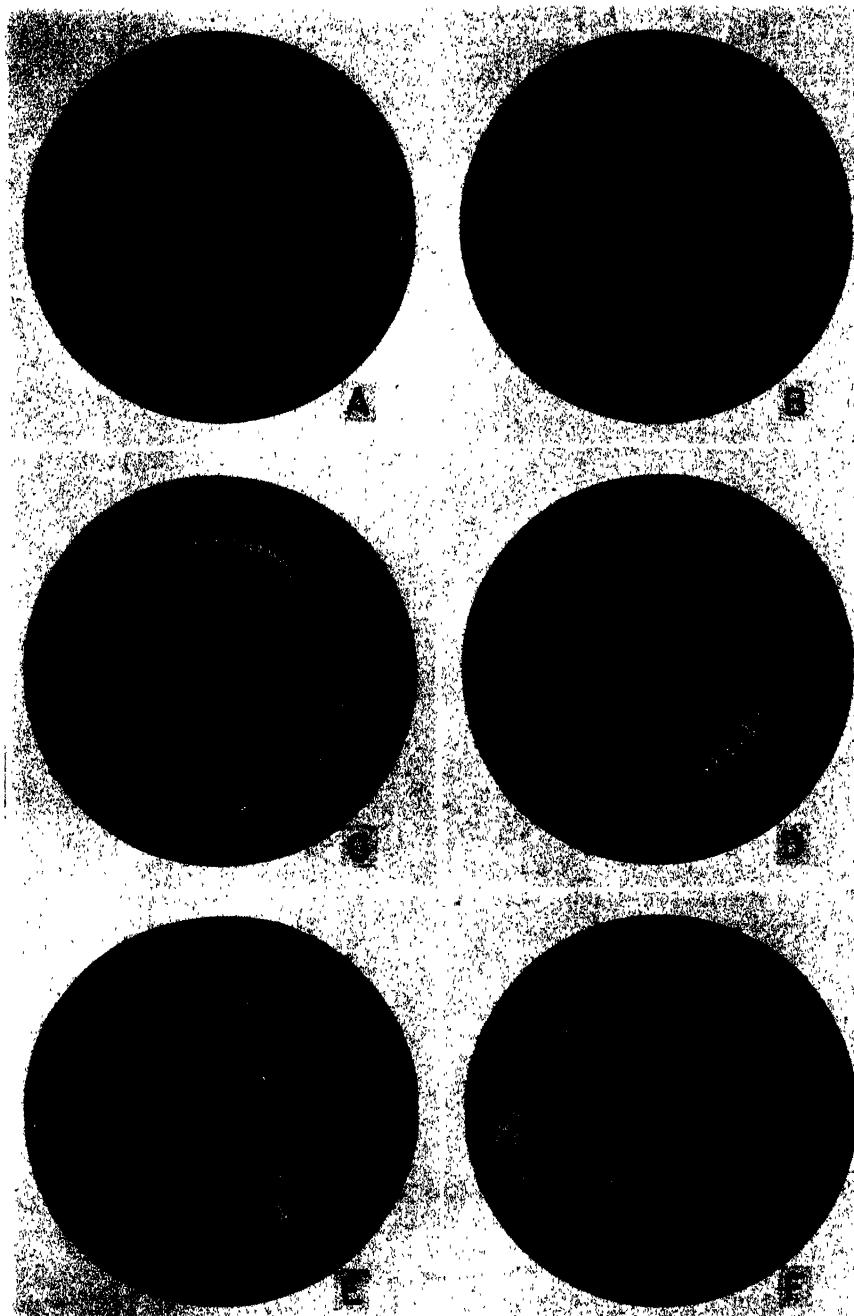


FIG. 2. The Fleming culture and selected derivative strains. *A*, stock the Fleming culture, NRRL 824; *B*, the Squibb strain, NRRL 1249; *C*, NRRL 1249.B21, the strain most widely used for penicillin production in surface culture; *D*, *E*, and *F*, substrains of 1249.B21 (see text discussion on pp. 84-86). *C* and *D* upon "E" medium; other cultures on Czapek's solution agar; all 10 days old. Incubation at 24°C.

reverse and agar in yellow shades. Colonies upon Czapek's solution agar with 1% added corn steeping liquor ("E" medium) 4 to 5 cm. in diameter in 10 days, closely furrowed in a radial pattern, heavy-sporing, dark gray-green in central area to artemisia green at colony margin, producing exudate; colony reverse and surrounding agar in dull yellow shades. Colonies upon malt extract agar 4 to 5 cm., plane, heavy-sporing, lighter blue-green near celandine green (Plate XLVII); no exudate produced; colony reverse dull yellowish.

Strain 824 is normally somewhat heavier-sporing than 1249, but in details of microscopic structure duplicates the latter strain (q. v.).

#### NRRL 1209—The Heatley Strain.

Colonies essentially like No. 824 upon each of the three media employed. Conidial apparatus as in No. 1249.

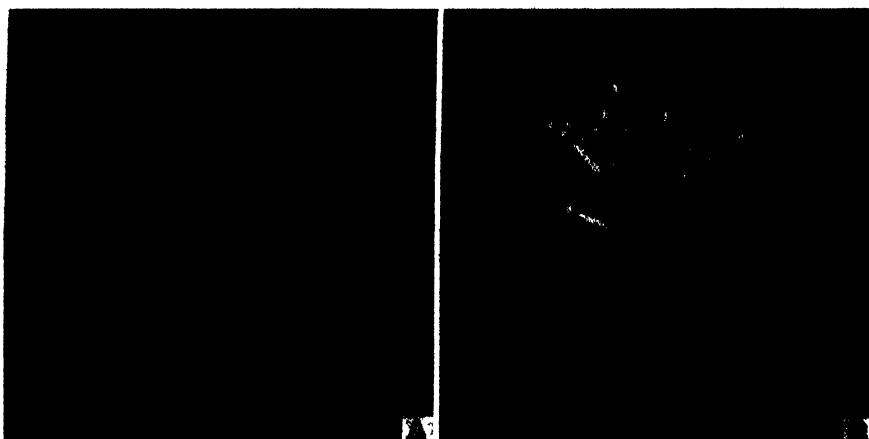


FIG. 3. A, typical penicillus of *P. notatum*, NRRL 1249, showing characteristic proportions and arrangement of parts; B, atypical penicillus in a substrain of 1249.B21 of the type illustrated in Fig. 2, D showing irregular arrangement of metulae and sterigmata,  $\times 750$ .

#### NRRL 1249—The Squibb Strain.

Colonies are essentially like those of No. 824 except generally lighter-sporing, often lighter in color, and show limited yellow exudate on Czapek solution agar and "E" medium.

Conidia arranged in tangled chains of variable and indefinite length, often reaching 75 to  $100\mu$ ; conidiophores generally unbranched, variable in length, ranging from 150 to  $400\mu$ , mostly 200 to  $300\mu$ , commonly 3.0 to  $3.4\mu$  in thickness; penicilli smooth throughout, variable in size, commonly 25 to  $35\mu$  in width in the sterigmata area, usually consisting of 2 to 3 metulae terminating the main stem, occasionally showing one or two side branches from 9 to  $12\mu$  x 2.5 to  $3.5\mu$ , occasionally longer; metulae usually borne in groups of 2 to 3, rarely more, from 10 to  $16\mu$  x 2.5 to  $3.0\mu$ , mostly 12 to  $13\mu$  x 2.5 to  $3.0\mu$ ; sterigmata commonly in groups of 3 to 5, from 8 to  $11\mu$  x 2.2 to  $2.8\mu$ ; conidia smooth, globose to subglobose, mostly 2.8 to  $3.5\mu$ , occasionally up to  $3.8\mu$ , individually pale green, *en masse* appearing darker. A typical penicillus is illustrated in Figure 3, A.

#### NRRL 1249.B21—Standard Surface Strain.

Colonies upon Czapek's solution agar in 3-point inoculation 4.5 to 5.0 cm. in diameter after 10 days at  $24^{\circ}\text{C}$ ., comparatively thin, close-textured, often somewhat zonate, showing conspicuous radial furrows, light-sporing, with outer zone of 1 to 3 mm. white, becoming

bluish green near celandine green or artemisia green (Plate XLVII) as conidia develop and mature; no exudate produced; colony reverse and surrounding agar in pale yellow shades. Colonies upon "F" medium radially furrowed (Figure 2, C), medium sporing, celandine green (Plate XLVII) to light grayish olive (Plate XLVI); no exudate; reverse in light tan shades. Colonies upon malt agar plane, comparatively heavy-sporing, pea green (Plate XLVII) slightly floccose at center; no exudate; reverse uncolored.

Conidial structures of the same general pattern as in No. 1249, but usually smaller; conidiophores variable in length from 50 to 300 $\mu$ , rarely branched, bearing 1 to 4 terminal metulae, 8 to 14 $\mu$  x 2.5 to 3.5 $\mu$ , each bearing 2 to 5 sterigmata, 7 to 10 $\mu$ , x 1.8 to 2.5 $\mu$ , definitely tapered at the apex; conidia globose to subglobose, smooth, ranging from 2.5 to 3.5 $\mu$ , pale green in color.

This high penicillin-producing strain, NRRL 1249.B21, differs from the parent strains 824 and 1249 in the following particulars: colonies are larger, light-sporing, lighter blue-green in color.

*Penicillin production in surface culture by selected strains of the Fleming mold, *P. notatum*, tested in August 1943 (Expt. 16) and again in September 1944 (Expt. 117)*

STRAIN NUMBER	PENICILLIN PRODUCTION							
	4th Day		5th Day		6th Day		7th Day	
	Penicillin O.u./ml.*	pH	Penicillin O.u./ml.	pH	Penicillin O.u./ml.	pH	Penicillin O.u./ml.	pH
Expt. 16								
824	50	7.0	70	7.6	75	7.6	63	7.9
1209	52	6.3	57	7.2	70	7.7	46	7.7
1249	98	7.1	103	7.6	81	7.8	74	8.1
1249.B21	53	6.0	80	6.8	133	7.7	118	7.8
Expt. 117								
824	78	7.3	98	7.6	92	7.9	79	8.2
1209	77	7.6	92	7.8	88	8.2	68	8.3
1249	92	7.3	120	7.6	114	8.0	103	8.2
1249.B21	102	6.8	150	7.3	166	7.7	162	8.1

\* Yields of penicillin are expressed as Oxford units per milliliter.

green in color, normally produce no exudate, and show comparatively little yellow color in reverse; conidial structures are generally smaller but remain typical in pattern.

A comparison of penicillin production in surface culture by the four strains that have just been considered is presented in Table I. Data from two representative experiments are included. The first of these, Experiment No. 16, was performed in August 1943; the second, No. 117, in September 1944. The production media employed in the two tests were of the same basic composition (see p. 77), but different lots of corn steeping liquor were employed. The reader will note that yields were consistently higher in the latter experiment. Variations of this magnitude are to be expected in comparative tests made at different times, and this is particularly true when different lots of steep liquor are employed. The most significant facts shown by these data are not the absolute yields of penicillin but the relative productivity of the different strains and the

progressive increase in yield from No. 824 to No. 1249, and from No. 1249 to No. 1249.B21. It is clearly demonstrated that more productive strains can be obtained through careful selection of variant types, and that, once obtained, such strains can be maintained at an essentially constant level of productivity.

### Single Spore Isolations

Strain 1249.B21 originated as a monosporous isolation from a culture of 1249 showing superior yields of penicillin. Attempts to obtain still more productive strains by this method have not succeeded. In the first of such experiments, 50 single spores were isolated from a good penicillin-producing stock, and the resulting cultures were tested for penicillin production in surface culture. All appeared essentially alike in culture and closely resembled the immediate parent. The average highest yield for duplicate flasks ranged from 149 O.u./ml. to 112 O.u./ml., with an over-all average of 128 O.u./ml. for the entire number. Spore suspensions were then prepared, and 16 monospore isolations were made from each of the three most productive lines and from that which had produced only 112 O.u./ml. to determine whether or not these differences would be inherited and sustained. In this second test, the average maximum yield of the isolates from two of the highest-producing lines, as well as from the lowest-producing line, of the previous experiment was 128 O.u./ml., or the same as for the entire 50 monosporous cultures included in the previous experiment. The average for the 16 isolates from the other culture was 132 O.u./ml. It seemed obvious that we were dealing with a comparatively stable clone in the good penicillin-producing stock from which the conidia had been obtained, and no further attempts were made to improve penicillin production by the isolation and testing of single-spore cultures.

### Substrains of NRRL 1249.B21

When cultivated upon nutrient-rich media, strain 1249.B21, like its progenitors, is subject to marked variation. Variation is principally in two directions. Most commonly encountered are variants which show progressively reduced sporulation, generally accompanied by reduced exudate and pigment formation, but which in varying degree retain the general growth rate and cultural pattern of 1249.B21. In sporulation cultures, or in production flasks, variants of this type appear as white, essentially sterile overgrowths of varying extent. In dilution plates, they appear as separate colonies characterized by comparatively loose, flocculent, white to light buff mycelia bearing very few conidia. In colonies in old agar plates they appear as sectors. Conidial structures, when present, are normally fragmentary and commonly atypical in pattern. Penicillin production is consistently lower than in the normal 1249.B21 type. Variants of this type under consideration are represented by "d" in Figure 4.

Another type of "white" variant, designated type "e", is illustrated in Figure 2, D. Examples have been isolated from streak plates inoculated from surface production flasks of 1249.B21. The type is characterized by closely felted, conspicuously furrowed colonies which in their basic pattern and texture strongly

suggest 1249.B21. Upon Czapek's solution agar they are light buff to cream in color and commonly show a pinkish tint—a character that is even more marked

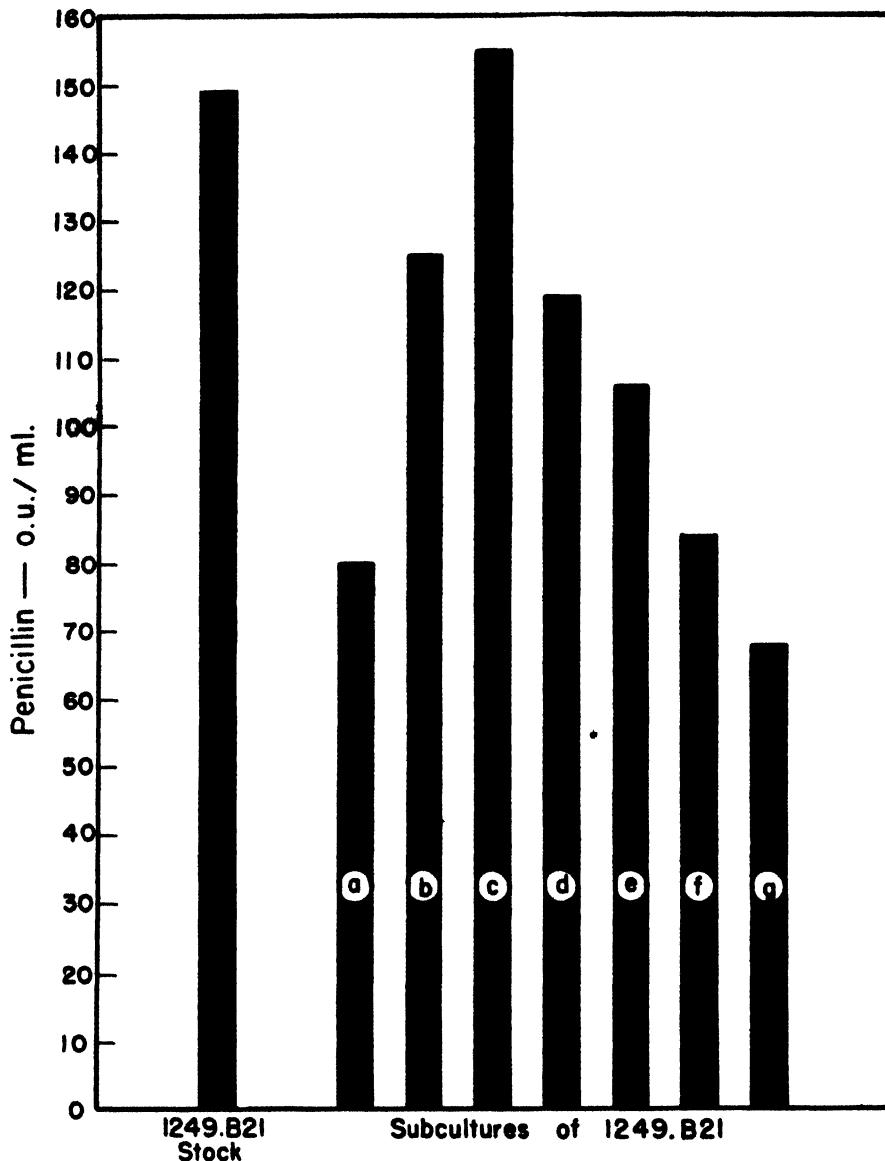


FIG. 4. Penicillin production in surface culture by NRRL 1249.B21 and subcultures of the same strain possessing different cultural characteristics. Values shown represent average maximum yields from duplicate flasks for one substrain of type "g," for two each of types "a," "b," "e," and "f," and for four each of types "c" and "d." See text discussion, pp. 84-86.

in colonies grown upon "E" medium. Conidial structures are widely scattered, and range from almost normal to very atypical and bizarre in pattern. One of

the latter is illustrated in Figure 3, B. Penicillin yields vary from approximately  $\frac{1}{2}$  to  $\frac{1}{3}$  those of the parent strain.

Other white variants, designated as type "g", are characterized by restricted, slow-growing colonies that produce few or no spores (Figure 2, E) and may or may not produce abundant exudate. Reasonably satisfactory surface production tests have been made by using as an inoculum liberal amounts of finely ground bran upon which the mold has been grown for approximately 10 days. As shown in Figure 4, penicillin production is low in comparison with the parent strain.

The "M" type of *P. notatum* (Fleming strain), isolated by Hansen and Snyder (23), represents a rapidly growing, non-sporulating form illustrated in Figure 2, F. Difficulties have been experienced by the writers in obtaining satisfactory inoculation of surface production flasks with the strain submitted by these investigators, but tests that have been made indicate that it is a poor penicillin producer.

Other variants of NRRL 1249.B21, occurring less frequently, are characterized by increased sporulation. These represent reversions in the direction of the original Fleming strain, and isolates showing all gradations between typical 1249.B21 and the Fleming culture (NRRL 824) have been observed. In general, penicillin production decreases progressively as sporulation becomes heavier.

Penicillin production by various substrains of 1249.B21 is compared with that of the parent stock in Figure 4. Type "a" represents a heavily sporing form differing little from No. 824; type "b" represents a somewhat less heavily sporing strain; while type "c" represents the strain 1249.B21 in its most characteristic cultural form. Types "d", "e", and "f" are characterized by progressively less sporulation, while type "g" represents a restricted colony producing no conidia upon most substrata. A general correlation between sporulation and penicillin production is thus found to occur among variant progeny of 1249.B21. Strains showing luxuriant sporulation give yields within the general range of No. 824; those showing more rapid vegetative growth and limited sporulation give higher yields; and, finally, those producing white, flocculent, poorly sporing or non-sporulating colonies again give lower yields. In working with strains derived from the Fleming culture, it is, therefore, extremely important to recognize the cultural characteristics of the most productive type represented by 1249.B21, and to avoid selection of cultures showing either greater sporulation on the one hand, or decreased spore production on the other.

• NRRL 832

None of the cultures descended from the Fleming strain, NRRL 824, have been found to produce satisfactory yields in submerged, or agitated culture (35, 39). For this method of production, entirely different strains have been employed. The culture first discovered (35) to give satisfactory yields when grown submerged was a strain of *P. notatum* contained in the mold collection of the Northern Regional Research Laboratory under the serial number 832. It has been universally employed in industry for the production of penicillin in deep tanks,

and only now is it beginning to be supplanted by more productive strains recently developed.

Culture NRRL 832 was brought to the Northern Regional Research Laboratory in the autumn of 1940 from the Thom Collection where it bore the serial number B-69. Dr. Thom had obtained it in 1936 through Dr. Paul Simonart, who brought it from Biourge's Laboratory as No. 69, labeled "*lacticus Mazé*." *Citromyces lacticus* Mazé and Perrier (Ann. Inst. Pasteur. 18: 558-559. 1904) was inadequately described as an ashy- or slate-blue mold. The generic designation indicates that it was monoverticillate. The mold actually present in the tube brought by Simonart was biverticillate and represented a deeply colored, rather restrictedly growing member of the *Penicillium notatum-chrysogenum* group. It is believed probable that it represents a contaminating mold which supplanted the original monoverticillate form during an extended period of laboratory cultivation.

There is no evidence which would indicate that strain 832 is historically related to the mold isolated by Fleming. Furthermore, it differs from the Fleming strain in several important cultural and morphological characters which will be noted in the subsequent discussion.

Strain 832 has been under intensive study in our laboratory for more than 2½ years. The strain represents an unusually stable member of the *P. notatum-chrysogenum* group, and only limited variation has been noted. Dilution cultures occasionally reveal colonies of variant character, and older colonies in agar plates sometimes develop sectors of distinctive nature. These have appeared most frequently in colonies at least 2 to 3 weeks old growing upon "E" medium. Such variants are reasonably stable, and, by exercising proper care in recultivation, can be maintained in culture in essentially constant form. Variants have not occurred in No. 832 in numbers comparable to those obtained from the Fleming strain, nor have they differed so markedly in penicillin production as in that classic form. A number of substrains have been isolated, however, which differ appreciably in appearance and to a limited degree in the amounts of penicillin that they produce. Some of these cultures will be compared with the stock strain with regard to cultural characteristics and penicillin production.

#### NRRL 832—Stock Culture.

Colonies upon Czapek's solution agar restricted, attaining a diameter of 3.0 to 3.5 cm. in 10 to 12 days at room temperature, with margin uniform or slightly lobed, up to 2 mm. deep in central area, strongly furrowed in a radial pattern, producing abundant yellow to light amber exudate that commonly collects into conspicuous droplets, heavy-sporing throughout except in extreme marginal zone 1 to 2 mm. wide, ranging in color from artemisia to lily green (Plate XLVII) or from Russian green to deep bluish grey-green (Plate XLIII), becoming slate olive in age (Plate XLVII); reverse in yellow to light brown shades with surrounding agar yellow. Upon "E" medium, colonies growing more rapidly, attaining a diameter of 4.0 to 4.5 cm. in 12 days, heavy-sporing, conspicuously furrowed, and in general presenting the same appearance as upon Czapek's solution agar (Figure 5, A). Colonies upon malt agar thin, plane, somewhat spreading, heavy-sporing, dark blue-green, no exudate.

Conidia are arranged in poorly defined, more or less divergent columns, commonly rang-

ing from 100 to 150 $\mu$  in length; penicilli are variable in dimensions and in complexity, occasionally showing one or more branches in addition to the main stem; conidiophores are variable in length, ranging from 125 to 350 $\mu$  x 3.0 to 3.6 $\mu$ , rarely larger or smaller; branches, when present, are of variable dimensions but commonly measure 10 to 15 $\mu$  x 3 to 3.5 $\mu$ ; metulae are borne in groups of 3 to 5 upon each branch, mostly measuring 8 to 10 $\mu$  x 3.0 to 3.5 $\mu$ ; sterigmata are commonly borne in groups of 4 to 7 and measure 6 to 8 $\mu$  x 2 to 3 $\mu$ , terminating

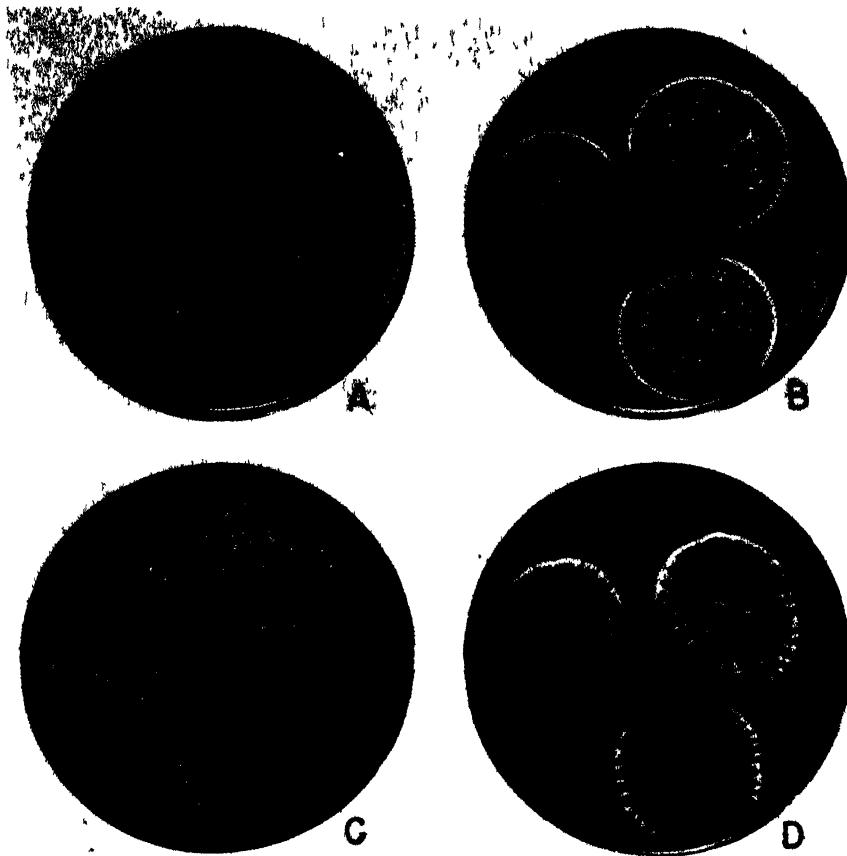


FIG 5 *Penicillium notatum*, NRRL 832, and three selected substrains grown upon "E" medium for 10 days at 24°C. A, the stock strain of 832, B, a substrain, 832 A<sub>2</sub>, characterized by increased exudate formation and deeper, loose-textured colonies; C, strain 832 A<sub>2</sub>(6), isolated as a sector from 832 A<sub>2</sub>, and characterized by abundant sterile mycelia which become red in age; D, strain 832 D<sub>1</sub>, isolated as a sector from 832 and characterized by thin, close-textured, heavy-sporing colonies

rather abruptly; conidia are elliptical to subglobose, mostly 3.0 to 3.6 $\mu$  x 2.2 to 3.0 $\mu$ , rarely as large as 4.2 x 3.6 $\mu$ .

In this strain branches (if present), metulae, and sterigmata are typically crowded together to form a comparatively short and compact spore-bearing structure that distinguishes it from each of the other cultures considered in the present report. Although NRRL 832 is believed to be correctly identified as *P. notatum* Westling, it is not entirely typical of this species if one uses as a standard for comparison the type strain of *P. notatum* (Thom 2541 · NRRL 821) received in 1911 by Dr. Thom from Westling. It apparently

represents a transitional form in the direction of *P. chrysogenum* Thom which is characterized by elliptical conidia, and in most strains by colonies of looser texture than the culture under consideration.

#### Strain 832.A<sub>2</sub>

This strain was isolated more than a year ago from a sporulation tube seeded with the stock culture of 832 in which small raised areas of brighter blue-green appeared. Spores removed from such areas when recultivated upon Czapek's solution agar gave rise to colonies differing from the parent culture in the following particulars: (1) They were deeper, more flocculent, ranging up to 3.0 to 3.5 mm.; (2) they were lighter-sporing, with central areas often yellowish and showing limited sporulations; and (3) they produced an unusual amount of yellow to light amber exudate which collected into large conspicuous drops. The strain is reasonably stable in culture.

Spore-bearing structures show wide variations in pattern, ranging from forms which duplicate those of the parent strain to other forms that are definitely atypical. The latter structures commonly fail to show either a definite separation into metulae and sterigmata or a regular sequence of branching; sterigmata are commonly borne irregularly near the terminus of the conidiophore, and sterigmata and conidia vary greatly in form and size.

#### Strain 832.A<sub>2</sub>(6)

This strain was isolated from a raised, light-sporing, pink-colored sector in a 3-week-old colony of 832 A<sub>2</sub> growing upon "E" medium in petri dish culture. Upon Czapek's solution agar colonies are restricted, conspicuously floccose, comparatively deep, predominantly yellow, producing abundant yellow exudate and very few conidial heads except in a narrow marginal zone. Upon "E" medium, growth is more luxuriant, but colonies are still restricted, strongly floccose and light-sporing, at first predominantly yellow but soon showing pink shades and in age becoming red, near daphne red, or Vernonia purple (Plate XXXVIII), usually showing a marginal zone of crowded fruiting structures (Figure 5, C'). This variant is stable in culture.

The spore-bearing apparatus varies in form and complexity and ranges in pattern from normal for NRRL 832 to atypical structures of the type noted for 832.A<sub>2</sub>.

#### Strain 832.D<sub>1</sub>

This strain was first observed as a somewhat depressed, heavy-sporulating sector in a giant colony of NRRL 832. When isolated and recultivated upon Czapek's solution agar, it formed restricted, comparatively thin, close-textured colonies that were heavy-sporing throughout and produced a limited amount of exudate. Upon "E" medium, colonies were larger but possessed the same general characters (Figure 5, D). The strain is reasonably stable in culture, but a definite tendency to revert to normal has been noted.

Spore-bearing structures are commonly smaller than the parent, 832, but are generally typical in pattern.

The production of penicillin in surface culture by the above strains is compared in Table II, wherein the results of two representative experiments are presented. In the first of these trials, strain 832.A<sub>2</sub> appears definitely superior to the parent culture, 832, while in the second trial, the two strains produce approximately equal yields. In both tests 832.A<sub>2</sub>(6) appears to be a good-producing strain equal to either of its forebears. In each case 832.D<sub>1</sub> proved to be a low-producing strain. The results of a representative experiment showing penicillin production in submerged culture by the above strains are presented in Table III. In this comparison, strain 832.B<sub>2</sub> was substituted for strain 832.D<sub>1</sub>, which was included in the surface culture comparison (Table II). The two

strains are culturally similar, and both show consistently low yields in surface culture. Of the four strains tested, 832.A<sub>2</sub> produced the highest yield; 832.A<sub>2</sub>(6) produced yields equal to those of 832; while 832.B<sub>2</sub> proved definitely inferior. In their survey of new isolates, Raper *et al.* (39) used strain 832.A<sub>2</sub> as the control and noted in Table IV that yields in submerged culture with this strain were slightly higher than for the stock culture of NRRL 832. In more recent and repeated tests, strain 832.A<sub>2</sub> has shown consistently higher yields, up to 25 to 30 per cent. While not great, the difference is well marked.

TABLE II

*Penicillin production in surface culture by NRRL 832 and three selected substrains in two separate experiments*

CULTURE	4TH DAY		5TH DAY		6TH DAY		7TH DAY	
	Penicillin O.u./ml.	pH						
832 Stock	49	7.4	60	7.8	52	8.2	40	8.3
832.A <sub>2</sub>	64	7.3	73	7.7	58	8.0	33	8.3
832.A <sub>2</sub> (6)	44	7.1	68	7.7	55	7.9	47	8.1
832.D <sub>1</sub>	35	6.8	27	8.0	20	8.2	14	8.4
832 Stock	76	7.4	74	7.9	74	7.9	38	8.1
832.A <sub>2</sub>	62	7.6	70	8.0	60	8.0	30	8.2
832.A <sub>2</sub> (6)	62	7.4	80	7.9	71	7.9	40	8.0
832.D <sub>1</sub>	41	7.1	46	8.0	26	8.2	23	8.3

TABLE III

*Penicillin production in submerged culture by NRRL 832 and three selected substrains*

CULTURE	3RD DAY		4TH DAY		5TH DAY		6TH DAY	
	Penicillin O.u./ml	pH	Penicillin O.u./ml.	pH	Penicillin O.u./ml	pH	Penicillin O.u./ml	pH
832 Stock	35	7.9	65	8.1	61	8.3	40	8.5
832.A <sub>2</sub>	47	7.9	92	8.0	83	8.3	50	8.4
832.A <sub>2</sub> (6)	37	8.1	62	8.0	75	8.2	52	8.4
832.B <sub>2</sub>	29	8.2	37	7.7	42	8.1	35	8.4

In marked contrast to subcultures isolated from the Fleming strain, subcultures of NRRL 832 have shown only limited correlation between sporulation and penicillin production. Yields with strains 832.D<sub>1</sub> and 832.B<sub>2</sub>, characterized by comparatively thin, close-textured but heavily sporing colonies, have been consistently low, and in this case we can associate a certain level of production with a definite cultural aspect. This is not true, however, of 832, 832.A<sub>2</sub>, and 832.A<sub>2</sub>(6). In this series there is a progressive decrease in sporulation and a progressive increase in vegetative growth and exudate formation without a substantial alteration in penicillin production. It is believed worthy of note, however, that the strain showing some increase in production is also characterized by intermediate spore production.

## NRRL 1950 AND DERIVATIVE STRAINS

*Penicillium notatum*, NRRL 1950, was isolated by the writers in August 1943 from a piece of molded Swiss cheese collected in Peoria, Illinois. It was one of a large number of strains belonging to the *Penicillium notatum-chrysogenum* group that was isolated from natural sources and tested for penicillin production by Raper, Alexander, and Coghill (39). In initial surface tests the strain produced yields of 75 to 85 O.u./ml., or about the same amount as the original Fleming strain, NRRL 824. In subsequent tests, surface yields have generally ranged from 70 to 100 O.u./ml. While this level of production was well above that of most of the new strains isolated and investigated in the survey (39), it was definitely lower than that of the best producing substrains of the Fleming culture, such as 1249.B21, which was already being employed for the commercial production of penicillin by the surface culture method.

When first tested in submerged culture in shaker flasks, NRRL 1950 produced substantially better yields than did the standard submerged strain, NRRL 832,

TABLE IV

*Penicillin production in surface culture by NRRL 1249.B21, 1950, 1978, and two substrains of 1978, A and B*

CULTURE	4TH DAY		5TH DAY		6TH DAY		7TH DAY	
	Penicillin O.u./ml.	pH	Penicillin O.u./ml.	pH	Penicillin O.u./ml.	pH	Penicillin O.u./ml.	pH
1249.B21	76	6.4	185	7.2	177	7.9	135	8.2
1950 Stock	98	7.3	139	7.7	103	8.0	81	8.3
1978 Stock	120	6.9	233	7.4	114	7.9	162	8.3
1978A	109	7.3	154	7.6	131	8.0	85	8.3
1978B	124	6.9	262	7.3	246	7.8	190	8.2

under comparable conditions. In subsequent trials extending over a period of six months, the average maximum yield for this strain was 68.5 O.u./ml., compared with 51 O.u./ml. for strain 832 (39).

Repeated attempts were made in this laboratory to secure natural variants of NRRL 1950 by dilution plating and by recultivation from and upon nutrient-rich substrata in the manner successfully employed with NRRL 1249. The strain was found to be unusually stable in culture, and no substrains were isolated which gave higher yields of penicillin than the parent stock.

Despite the fact that we did not secure more productive substrains, the new isolate, NRRL 1950, in its original form appeared to have definite potentialities as a submerged culture. For this reason, it was one of the strains submitted by the Northern Regional Research Laboratory to Dr. G. W. Beadle of Stanford University<sup>a</sup> in April 1944 to be irradiated in the hope of securing higher penicillin-

<sup>a</sup> Early in 1944 the Office of Production and Research Development of the War Production Board set up a project at Stanford University under the direction of Dr. G. W. Beadle designed to investigate the possibilities of producing, by ultraviolet and X-ray radiation, mutations characterized by increased penicillin production.

producing cultures. Subsequent to this, Dr. Beadle returned to us a mutation (his No. 1982 = NRRL 1978) obtained by irradiating conidia with ultraviolet light. In surface production tests this strain gave yields of penicillin equal to or exceeding those of 1249.B21. By selective recultivation of this strain from production flasks, substrains have been isolated which show some further improvement.

### NRRL 1950

Colonies upon Czapek's solution agar in 3-point inoculation, 4.5 to 5.0 cm. in diameter after 10 days' incubation at room temperature, commonly floccose and light-sporing in central area, often light yellow buff, comparatively deep, up to 2 to 3 mm., radially furrowed, thinning toward the colony margin with outer zone velvety, heavy-sporing, at first light celandine green through artemisia green and lily green (Plate XLVII) to near castor gray (Plate LII) in age; usually producing abundant yellow exudate (Figure 7, A); colony

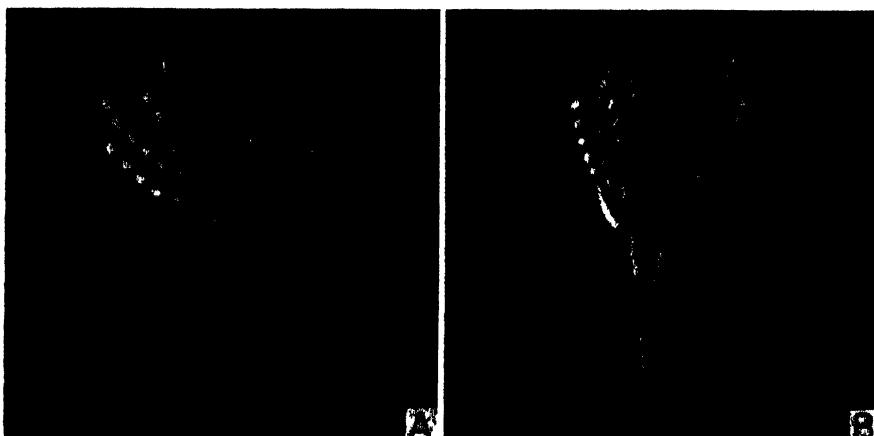


FIG. 6. A, typical conidial structure of *P. notatum*, NRRL 832; B, typical conidial structure of *P. notatum*, NRRL 1950. Note particularly the compact penicillus and elliptical conidia which characterize the former; and the small, loose penicillus and subglobose conidia characteristic of the latter.  $\times 750$

reverse in yellow to light brown shades with surrounding agar yellow. Colonies upon "E" medium 5.0 to 6.0 cm. in diameter in 10 days, velvety, heavy-sporing, in green-blue shades when young (Plate XLVIII), showing conspicuous radial furrows, limited exudate; yellow to light amber; reverse in pale yellow shades. Colonies upon malt agar 5.0 to 6.0 cm. in diameter, spreading rapidly, thin, plane, light celandine to artemisia green (Plate XLVII), no exudate; reverse in light yellow shades.

Conidia arranged in loose, poorly-defined columns of indefinite length but often reaching  $100\mu$ ; conidiophores unbranched, variable in length up to 300 to  $400\mu$ , commonly 2.7 to  $3.2\mu$  in diameter. Penicilli smooth throughout, characteristically small, often consisting of only 2 or 3 metulae, bearing sterigmate cells as shown in Figure 6, B, rarely showing side branches; metulae commonly ranging from  $12$  to  $18\mu$   $\times$   $2.2$  to  $2.8\mu$ ; sterigmata thin and variable in length from  $7.5$  to  $11\mu$ ; confidia smooth, globose to subglobose,  $2.5$  to  $3.2\mu$  in diameter, mostly about  $2.8\mu$ , light blue-green in color.

In surface culture, upon the standard production medium employed in this study, maximum yields of penicillin ranged from 70 to 100 O.u./ml. In submerged culture in shaken flasks employing the standard test medium listed on p. 78, maximum yields ranged from 60 to 90 O.u./ml.

Physiologically as well as morphologically, NRRL 1950 appears to be exceptionally stable. It is characterized particularly by its abundant and comparatively small fruiting structures which commonly consist of 2 to 3 metulae, each bearing a limited number of sterigmata.

### NRRL 1978

Strain 1978 represents an ultraviolet-induced mutation of NRRL 1950 produced by Dr. Beadle and associates at Stanford University and sent to us as No. 1982 early in the summer

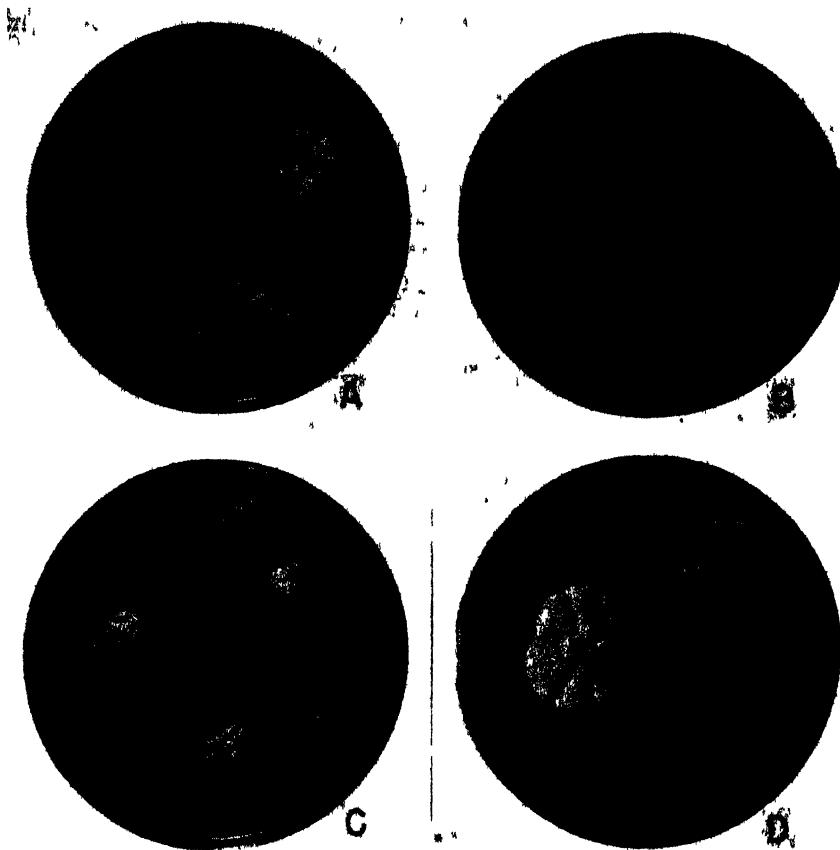


FIG. 7. *Penicillium notatum*, NRRL 1950, and derivative strains on Czapek's solution agar at 10 days, 24°C. A, stock culture of 1950; B, stock culture of 1978, an ultraviolet-induced mutation of 1950 produced by Dr. G. W. Beadle and associates at Stanford University; C, NRRL 1978.B, a substrain of 1978; and D, a substrain of 1978.B, representative of type 1978.B. See discussion in text, p. 94.

of 1944. The mutant differs materially from the parent strain in cultural appearance, but not in details of morphology. Sporulation is generally less heavy and colonies are lighter blue-green upon most substrata. For example, upon Czapek's solution agar, colonies range from light celandine green (Plate XLVII) to a shade near storm gray (Plate LII) when young, approaching Quaker drab to mouse gray (Plate LI) in age. Exudate formation is reduced (Figure 7, B). Conidial structures are of the same general pattern as in the parent, 1950. Yields of penicillin in surface culture range between 50 to 80 per

cent higher than those of the parent under like conditions, while yields in submerged culture are at the same level, or even lower, than from the parent stock.

By streaking plates with spores taken from surface production flasks of 1978 showing high yields of penicillin, and subsequently making isolations from the colonies that developed, two types of substrains were obtained. The first of these, designated 1978.A, produced somewhat heavier sporing, increased exudate-forming colonies that generally showed somewhat lower yields of penicillin than the parent 1978 when tested in surface culture. The second, 1978.B, was characterized by less heavily sporing colonies and has consistently shown slightly higher surface yields than 1978 (Figure 7, C). The results obtained in a representative test are presented in Table IV.

Strain selections, in turn, have been made from dilution plates seeded with spores of 1978.B. Colonies of three general types have been isolated. The first of these is essentially like the immediate parent; the second is characterized by decreased sporulation and a lighter blue-green color; while the third produces almost no spores, and is light yellow-buff in color (Figure 7, D). Of the three types, designated No. 1978.B<sub>1</sub>, 1978.B<sub>2</sub>, and 1978.B<sub>3</sub>, respectively, the first produces yields of penicillin approximating those of 1978 and less than 1978.B; the second equals or exceeds the parent, 1978.B, while the last type yields only about half the amount produced by 1978.B, and reaches this level at 6 to 8, rather than 4 to 6, days due to the slowness of mat formation that results from inadequate inoculation.

Penicillin production in NRRL 1950 and in the various derivatives of this strain that have been discussed above is graphically compared in Figure 8. With the single exception of 1978.B<sub>3</sub>, the results shown are taken from a single experiment and the values listed represent the average maximum penicillin production for duplicate flasks for each substrain tested. While actual penicillin yields obtained in other experiments have differed from those shown, the relative productivity of the different strains and types has remained essentially constant in all tests (cf. Table IV and Figure 8). Experiments with No. 1978 and its derivatives have not been in progress long enough to gauge accurately the stability of these different strains. Sufficient work has been done, however, at Stanford University and in this Laboratory, to establish beyond doubt that in the progeny of strain 1950, as in that of the Fleming strain, the most productive substrains are characterized by somewhat reduced sporulation, which is reflected in a lighter blue-green colony color, and by reduced exudate formation. Similarly, as in the Fleming culture, substrains characterized by light sporulation and limited exudate formation which produce high yields of penicillin in surface culture fail to show a corresponding increase in productivity in submerged culture.

In industry, strain NRRL 1950 has been successfully used for producing penicillin by cultivating the mold upon sterilized and enriched bran. The more productive substrains subsequently developed at Stanford University and at the Northern Regional Research Laboratory have recently been released to penicillin producers and may contribute materially to the production of the drug by both the surface and the bran methods.

## NRRL 1951 AND DERIVATIVE STRAINS

*Penicillium chrysogenum*, NRRL 1951, was isolated by the writers in July 1943 from a small patch of blue-green mold growing at the point of stem detachment on a cantaloupe contributed by a housewife in Peoria, Illinois. In initial surface tests, yields of penicillin up to 70 to 75 O.u./ml. were obtained in contrast to 125 O.u./ml. for the control strain, NRRL 1249.B21. Maximum yields of 45 to 50 O.u./ml. were obtained when the strain was first tested in shaken flasks. The latter yields were approximately equal to those obtained in submerged

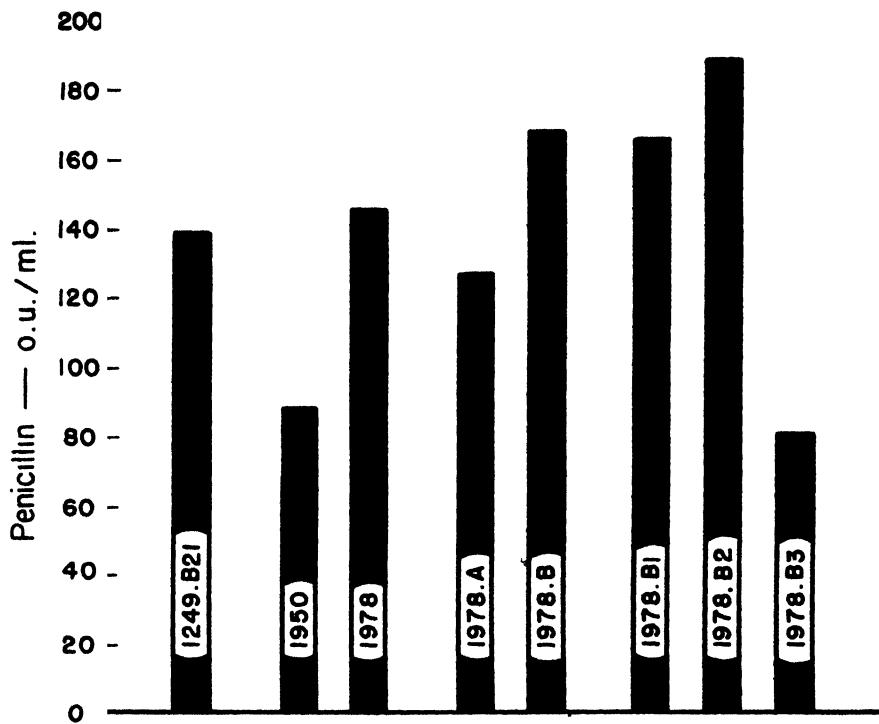


FIG. 8. Comparative penicillin production in surface culture by NRRL 1249.B21, 1950, and substrains derived from the latter culture. See text discussion, p. 94. Values plotted represent average maximum yields in duplicate flasks of single strains except as follows: for 1978.B<sub>1</sub>, average maximum yields in duplicate flasks of three substrains; for 1978.B<sub>2</sub>, six substrains; and for 1978.B<sub>3</sub>, four substrains.

culture with the control strain, NRRL 832. Repeat runs were made, and in the second of these tests, triplicate flasks showed potencies of from 80 to 90 O.u./ml. at 5 days, or approximately 75 per cent more than the control 832, tested under identical conditions. The culture appeared to be outstanding. Penicillin yields were unusually good, and the titre of the broths remained high through the sixth and seventh days.

In order to recover and perpetuate this high-yielding strain, loopfuls of the broth and mycelium (in pellet form) from the highest yielding flask were streaked upon Czapek's solution agar plates. Except for two conspicuous sectors, the

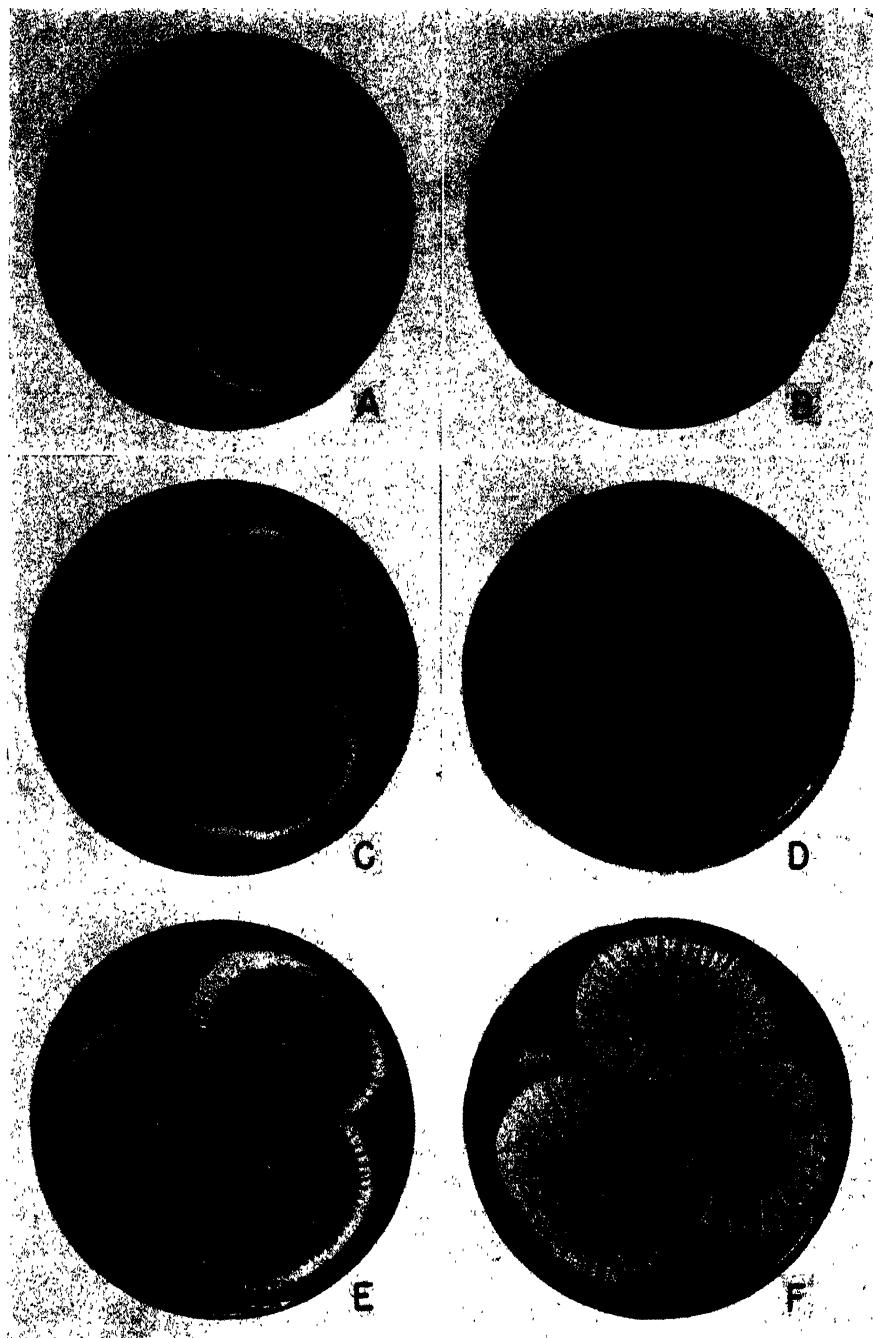


FIG. 9. *Penicillium chrysogenum*, NRRL 1951, and derivative strains on "E" medium, 10 days old, incubated at room temperature. A; stock culture, NRRL 1951; B and C, 1951.B and 1951.C, respectively, substrains of 1951; D, E, and F, 1951.B10, 1951.B25, 1951.B15, respectively, substrains of 1951.B. See text discussion, p. 98, and Fig. 10.

resulting growth duplicated, in appearance, the loose-textured, heavily sporing parent (Figure 9, A). The first of these sectors was characterized by abundant yellow mycelium and exudate and produced comparatively few spores; it was isolated and designated 1951.B (Figure 9, B). The second sector differed from the first in being closer textured and somewhat heavier-sporing; otherwise, the two were strikingly similar. It was isolated and designated 1951.C (Figure 9, C). A third isolation, designated 1951.A, was made from the heavy-sporing, loose-textured growth which constituted the greater portion of the streaks. Penicillin production in surface culture by these substrains was compared with NRRL 1249.B21 two weeks later with the results shown in Table V.

TABLE V

*Comparison of penicillin production in surface culture by NRRL 1249 B21 and three substrains of NRRL 1951*

CULTURE	4TH DAY		5TH DAY		6TH DAY		7TH DAY	
	Penicillin Ou/ml	pH	Penicillin Ou/ml	pH	Penicillin Ou/ml	pH	Penicillin Ou/ml	pH
1249 B21	100	6.9	142	7.5	128	7.9	70	8.3
1951 A	23	7.6	25	7.9	27	8.0	20	8.2
1951 B	53	6.5	90	7.3	135	7.9	128	8.0
1951 C	74	7.2	104	7.7	154	7.9	132	8.2

TABLE VI

*Comparison of penicillin production in submerged culture by NRRL 832 and two substrains of NRRL 1951*

CULTURE	3RD DAY		4TH DAY		5TH DAY		6TH DAY	
	Penicillin Ou/ml	pH	Penicillin Ou/ml	pH	Penicillin Ou/ml	pH	Penicillin Ou/ml	pH
832	23	8.1	34	7.9	49	8.0	45	8.3
1951 B	26	7.9	53	8.1	76	8.0	69	8.4
1951 C	18	8.0	29	8.1	47	8.0	51	8.3

The results were most encouraging. Strain 1951.B approached 1249.B21 as a producer of penicillin in surface culture, while 1951.C actually surpassed it. For the first time strains had been found which differed markedly from any of the derivatives of the Fleming strain, yet matched the best of these for the surface production of penicillin. One disadvantage was foreseen—maximum yields in each case were produced one day later than with 1249.B21. The experiment was repeated, and the earlier results were wholly confirmed. Because of its low productivity, strain 1951.A was considered to offer little promise and was eliminated from further consideration.

Strains 1951.B and 1951.C were then tested for their capacity to produce penicillin in submerged culture. The standard survey medium was employed, and inoculated flasks were incubated on Ross-Kershaw shakers at 24°C. Results are presented in Table VI.

Strain 1951.C produced yields of penicillin equal to NRRL 832, while 1951.B produced yields 50 per cent in excess of the latter.

Based upon the results reported above for surface- and submerged-culture tests, together with other data of a confirmatory nature, the writers decided to investigate strain 1951.B as thoroughly as possible in the hope that even more productive substrains might be isolated.

As an initial approach, a spore suspension was prepared from a stock culture of 1951.B. Fifty single spores were isolated and the resulting cultures were tested for penicillin production in surface culture. Differences in yield were obtained from these monospore isolates, but none produced yields either substantially greater or markedly less than the parent stock which had been under investigation. The ultimate possibilities in this approach were not denied, but it was felt that other methods offered greater promise, at least for the immediate future.

In further attempts to secure more productive substrains, dilution plates in Czapek's solution agar were prepared from a 3-week-old culture of 1951.B growing upon a sporulation medium. The slant from which the spores were taken was somewhat mottled in appearance due to an admixture of numerous areas possessing different growth characteristics. The spore suspension was made as representative as possible of the entire culture. Following an incubation period of 10 days at room temperature, subcultures were made from 32 of the resulting colonies. While some cultures of intermediate or otherwise different character were isolated, the majority could be grouped into three general types as illustrated in Figure 9, D, E, and F: Type I, cultures characterized by velvety, heavy-sporing, deep blue-green colonies producing limited to abundant yellow exudate (Figure 9, D); type II, cultures characterized by rather loose-textured, medium- to light-sporing colonies in yellow-green shades (Figure 9, E); and type III, yellow-floccose colonies producing almost no spores and an abundance of yellow exudate (Figure 9, F).

The cultures thus isolated were first tested for their capacity to produce penicillin in surface culture. Comparative yields are presented graphically in Figure 10 where the values shown represent the highest average production for duplicate flasks. Yields are compared with that of the control strain 1249.B21. Cultures of types I, II, and III, as well as the two unclassified isolates to be described presently, are shown as differently cross-hatched columns. A striking correlation between cultural characteristics and penicillin production was observed. The highest yielding strains were consistently of type II, while yields in types I and III were approximately equal, but substantially lower than those of type II. Isolate 1951.B21, producing low yields, was characterized by velvety, heavy-sporing, yellow-green colonies showing conspicuous sectors. Isolate 1951.B28, producing good yields, ~~was~~ characterized by restricted, medium-sporing colonies, light celandine green (Plate XLVII) in color.

Facilities were not available for testing all of the isolates in submerged culture, and an arbitrary selection was made. Results are shown in Figure 11 where listed values represent the average yields of triplicate flasks. Each of five isolates belonging to type III produced good yields of penicillin, as did also

1951.B10, a heavy-sporing strain of type I, which had not been outstanding in the earlier surface test (Figure 10). Isolates 1951.B4 and 1951.B10 appeared strikingly similar in agar plate cultures, and in surface tests they had produced approximately equal amounts of penicillin. In submerged culture, penicillin production by 1951.B4 was quite low. Obviously the two strains possessed marked physiological differences. Isolate 1951.B28, which differed from type II but produced good surface yields of penicillin, gave low penicillin yields when

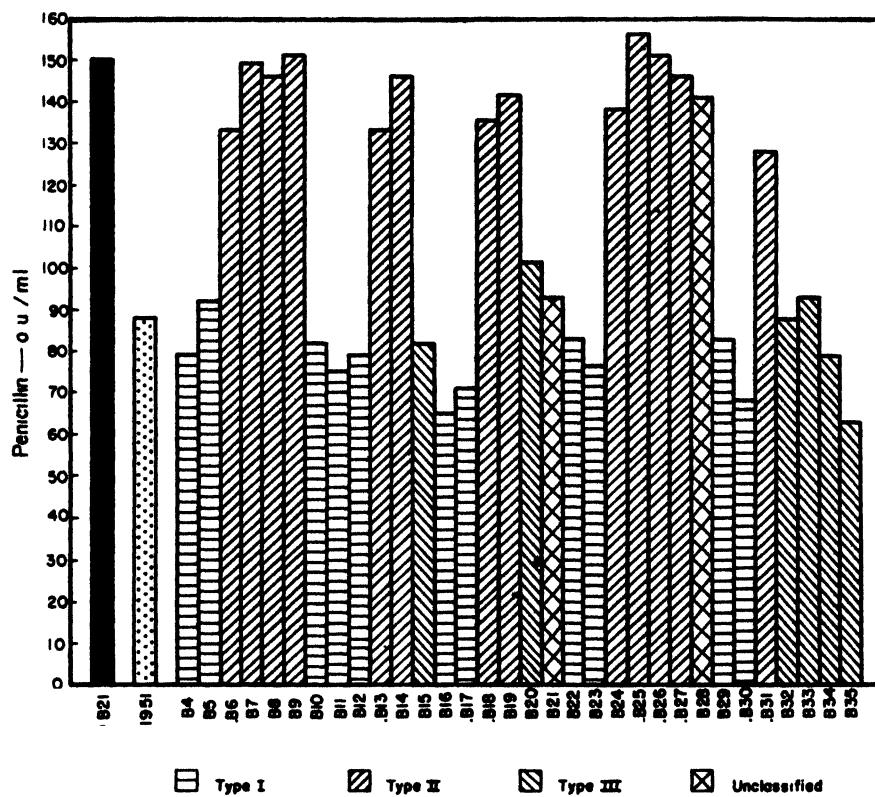


FIG. 10. Production of penicillin in surface culture by NRRL 1951, 32 substrains of 1951.B, and the control culture, 1249.B21. Note the general correlation between type of culture and yields of penicillin. Values plotted represent average maximum yields of duplicate flasks. See text discussion, p. 98.

grown in submerged culture. While no control flasks inoculated with NRRL 832 were included in this experiment, this standard submerged-culture strain under the same conditions would be expected to produce a maximum of 60 to 65 O.u./ml., or approximately one-half the yields of the best substrains of 1951.B.

While other subcultures that approached this strain were isolated, it was considered significant that isolate 1951.B25 gave the highest yield of penicillin in both the surface and the submerged tests. Quite naturally, our attention was directed toward this strain as the possible source of still higher producing forms.

In laboratory culture, NRRL 1951.B25 is quite unstable and tends to dissociate readily in the same manner as the parent strains 1951.B and 1951. Despite this characteristic, the writers have succeeded in maintaining the strain at a constant level of productivity over a period of 8 months (1) by lyophil preservation (40) and (2) by infrequent recultivating upon agar media. In both cases special precautions have been taken to employ an inoculum that is representative of the whole culture.

Striking contrasts in cultural characteristics and in details of microscopic structure exist between the parent strain, NRRL 1951, and such highly productive substrains as 1951.B25. These differences will now be considered.

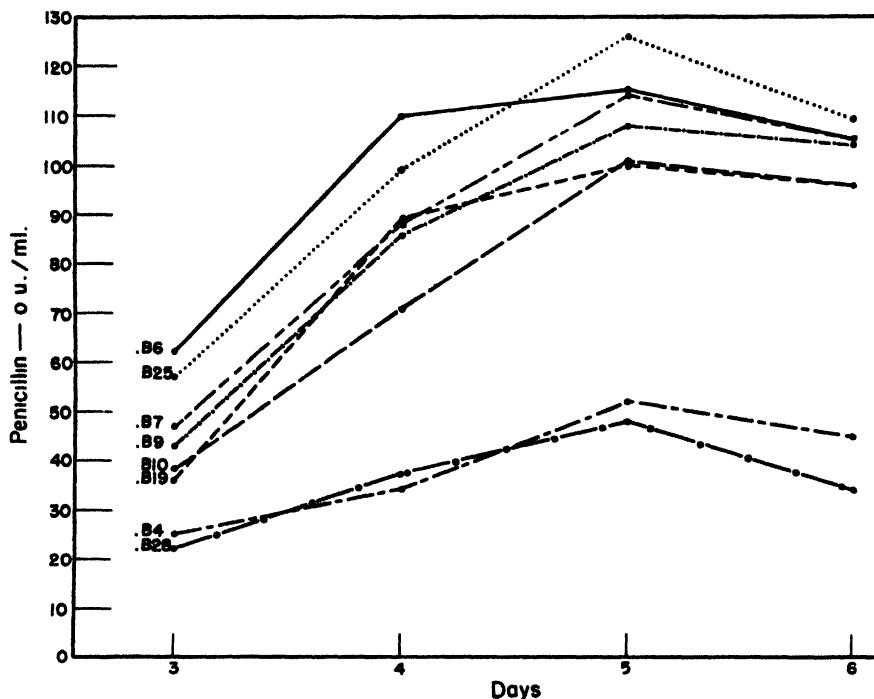


FIG. 11. Production of penicillin in submerged culture by eight selected substrains of 1951.B. Values plotted represent average maximum values for triplicate flasks. Type of culture and comparative yields of penicillin in surface tests are shown in Fig. 10.

#### NRRL 1951

Colonies upon Czapek's solution agar in 3-point inoculation 4.5 to 5.0 cm. in diameter in 10 days at room temperature, loose-textured, not floccose, comparatively deep, up to 2 mm., heavy-sporing throughout except in a narrow marginal zone, at first yellow-green, near pistachio green to American green (Plate XLI), becoming blue-green, near Russian green, or dark Russian green (Plate XLII) in age; reverse in dull yellow-green shades, occasionally showing brown, surrounding agar yellow. Colonies upon "E" medium growing more rapidly, heavier-sporing and producing less exudat<sup>+</sup> than upon Czapek's agar; otherwise essentially similar. Colonies upon malt agar spreading rapidly, plane, heavy-sporing, at first yellow-green, near pea green to sage green (Plate XLVII), becoming slate olive (Plate XLVII) in age, reverse uncolored.

Conidial heads large, usually characterized by 3 to 5 divergent, or radiating, columns of spores arising from a large and much branched penicillus of the general type illustrated in Figure 12, A; spore columns variable in length, commonly up to  $350\mu$  by 15 to  $20\mu$  in diameter; conidiophores long, generally unbranched except in the terminal area, supporting penicilli which often show one or more primary branches, each supporting 2 or more secondary branches which in turn bear from 2 to 5 metulae, smooth-walled throughout; primary branches variable in length, commonly up to 20 to  $25\mu$  in length by  $3.0$  to  $3.5\mu$  in diameter; secondary branches from  $10$  to  $15\mu$  x  $2.5$  to  $3.0\mu$ ; metulae  $10$  to  $12\mu$  x  $2$  to  $3\mu$ , occasionally larger, sterigmata mostly  $8$  to  $10\mu$  x  $2.0$  to  $2.5\mu$ ; conidia elliptical, rarely subglobose, mostly  $3.0$  to  $3.5\mu$  x  $2.5$  to  $3.0\mu$ , occasionally larger, smooth-walled, pale blue-green in color.

Agar plate and tube cultures of NRRL 1951 are comparatively stable and upon recultivation normally reproduce the same cultural picture. Penicillin production, however, is extremely variable. Under constant conditions and upon similar media, surface yields have ranged from 40 O.u./ml. up to 90 O.u./ml. in different experiments. In submerged culture, yields have ranged

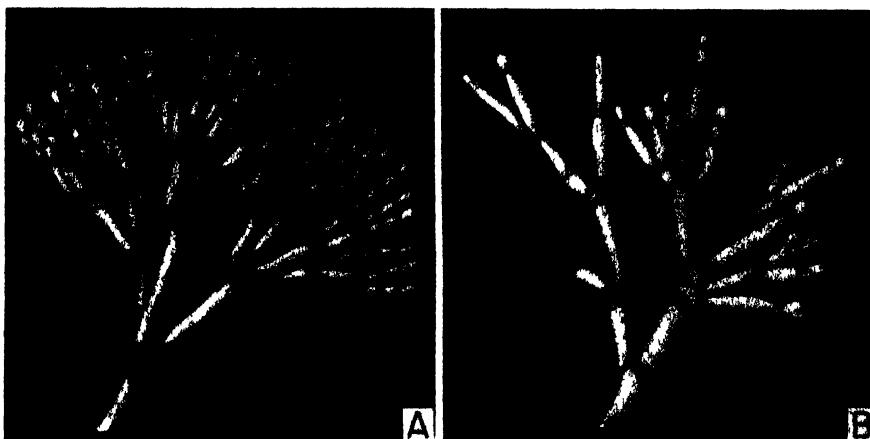


FIG. 12. A, Typical penicillus of NRRL 1951; B, characteristic penicillus of NRRL 1951, B25. Note the irregularity in the arrangement, form, and size of parts in the latter structure.  $\times 750$ .

from 35 O.u./ml. to around 90 O.u./ml. No definite explanation can be offered, but it is suspected that in flasks inoculated with the so-called stock culture, different types of growth gain the ascendancy in different tests and so account for these differences in yield. In support of this view is the fact that high yielding strains 1951.B and 1951.C were isolated from a submerged culture distinguished by its high titre.

In cultural appearance and the details of its microscopic structure, NRRL 1951 is fairly representative of a type of mold not infrequently isolated from soil and other natural habitats. It is our belief that the type is most satisfactorily diagnosed as *P. chrysogenum* Thom, although it does not duplicate in appearance the type strain of this species, Thom's No. 26 (NRRL No. 807). NRRL 1951 is, on the whole, a looser-textured, more robust strain that produces larger penicilli and conspicuously elliptical conidia. In certain details of cultural behavior and microscopic structure it resembles a strain obtained from the

Thom collection as *P. baculatum* Westling (Thom's No. B-497: NRRL 843). It was not assigned to this species since, in the examination of large numbers of isolates from natural sources (39), no definite line of separation between *P. chrysogenum* and *P. baculatum* could be found. It is considered probable that the latter species, as it was originally described by Westling and as it has been subsequently interpreted by Thom, represents essentially a comparatively coarse, large-spored type of *P. chrysogenum*.

#### NRRL 1951.B

In cultural appearance (Figure 9, B) and in details of morphology, strain 1951.B bears a striking resemblance to 1951.B25 subsequently to be considered. In parallel production tests, yields of penicillin are, as a rule, somewhat lower than in the latter strain.

#### NRRL 1951.C

Typically this strain differs from 1951.B in showing closer-textured, somewhat heavier-spored colonies (Figure 9, C), usually showing less yellow mycelium and producing less exudate. It produces good yields of penicillin in surface culture and has been made available to producers for this purpose. This stock and substrains derived from it are currently being used successfully for commercial production in some plants. This strain, and also 1951.B25, have been found particularly useful in situations where it is not practicable to control temperature at 24°C., the optimum for NRRL 1249.B21.

#### NRRL 1951.B25

Colonies upon Czapek's solution agar in 3-point inoculation 4.0 to 5.0 cm. in diameter after 10 days' incubation at 24°C., with central areas closely felted, light-spored, predominantly in light yellow-green shades, producing abundant yellow to amber exudate, showing a limited number of conspicuous radial furrows, marginal area at first white, subsequently developing conidial structures and becoming light blue-green in color near stone green to bluish gray-green (Plate XLII); reverse in yellow shades, becoming light brown in age, surrounding agar yellow. Colonies upon "E" medium growing more rapidly, close-textured, heavier sporing, in yellow-green shades near sage green to andover green (Plate XLVII), closely furrowed in a radial pattern (Figure 9, E), producing abundant amber exudate in small droplets; reverse in pale yellow shades, agar similarly colored. Colonies upon malt agar spreading, 6.0 to 7.0 cm. in diameter in 10 days, plane, or showing few wrinkles in central area, thin, not felted but consisting of a dense stand of conidial structures arising from the agar surface; reverse in dull yellow shades.

Conidial structures characteristically atypical in pattern, not producing well-defined columns of conidia on any medium. Penicilli often borne upon short conidiophores arising from trailing hyphae, irregular in pattern and ranging from very simple to quite complex, branching irregularly; metulae varying greatly in length and often poorly defined, sterig-mata sometimes arranged in clusters, often not, varying greatly in form and size; conidia extremely variable in shape and dimensions, subglobose to conspicuously elliptical. No single figure can adequately depict the irregular and often bizarre character of these structures, but the difference between these conidial heads and those of the parent NRRL 1951 is suggested in Figure 12, B, where a representative structure is illustrated.

In repeated comparative tests NRRL 1951.B25 has produced yields of penicillin equal to those of 1249.B21 in surface culture. Maximum yields generally

appear at least one day later, but this lag period can be shortened by increasing the amount of inoculum. Luxuriant sporulation does not occur upon most laboratory media or upon the sporulation medium recommended for NRRL 1249.B21 by Moyer and Coghill (34). Such spore production can be obtained, however, upon moist bread or other cereal products carefully sterilized, or, as Dr. Moyer has found, upon sporulation media to which additional NaCl is added. Like 1951.C, the strain is not particularly sensitive to temperature, and good penicillin yields can be obtained above 24°C., which is optimum for 1249.B21. Strains derived from this culture are being used successfully for the commercial production of penicillin in surface culture.

NRRL 1951.B25 warrants special consideration as a producer of penicillin in submerged culture. In the initial test, yields up to 136 O.u./ml. were obtained in one of triplicate flasks at 5 days, while the average maxima of the three was 126 O.u./ml. Since that time the stock strain has been tested in shaken flasks again and again upon our standard test medium (see p. 78), and in some instances yields in excess of 150 O.u./ml. have been realized. In other cases maximum yields have not exceeded 100 O.u./ml. Reasons for this fluctuation have not been discovered, although a number of possible influencing factors, such as the amount of aeration, the quantity of inoculum, etc., have been investigated. Composition of the test medium and the temperature of incubation have remained constant. Despite variations in yield, there has been no evidence of either increased or decreased productivity by the stock strain during the period that it has been under investigation, since high and low potency series have been scattered throughout the more than 20 tests that have been run. Whenever comparative tests in shaken flasks have been run, penicillin yields in 1951.B25 have been, as a rule, approximately double those of NRRL 832, which for many months was used exclusively for the production of penicillin in submerged, or tank, culture. Strain 1951.B25 was made available to other research laboratories and manufacturers several months ago and has become the subject of wide study. It is now being used in some plants for the commercial production of penicillin by the submerged method, and its adoption in other establishments is foreseen for the near future.

#### Substrains of 1951.B25

Strain 1951.B25, like the parent strain 1951.B, is quite unstable in culture, and among the colonies developing in either streak or dilution plates several different types can usually be distinguished (Figure 1, B). When isolated and recultivated, these substrains in turn tend to vary, and the process seems to be unending. Any given substrain, however, can be perpetuated satisfactorily by lyophil preservation of a spore suspension that is representative of the total culture; or by recultivation upon agar plates or in tubes, using a mass inoculum containing conidia drawn from an extensive area of the parent culture. Dozens of these substrains have been tested for penicillin production in both submerged and surface culture, but, as previously noted, none have been found to surpass it in penicillin production.

Some of these substrains of 1951.B25 are of interest from a mycological point

of view, since they illustrate the wide diversity of cultural types which can arise from a single parent stock, itself of variant origin. In Figure 13 are illustrated four substrains isolated from a single streak culture of 1951.B25 inoculated from a 1-week-old submerged production flask that showed a maximum yield of 158 O.u./ml. at 5 days. In Figure 14 are shown average maximum penicillin yields in surface and submerged culture by the same strains.

Substrain *A* is loose-textured, heavy-sporing, and resembles rather closely the stock strain of 1951. Surface yields were quite low, while submerged yields reached 90 O.u./ml. This and other isolates of the same type are of special

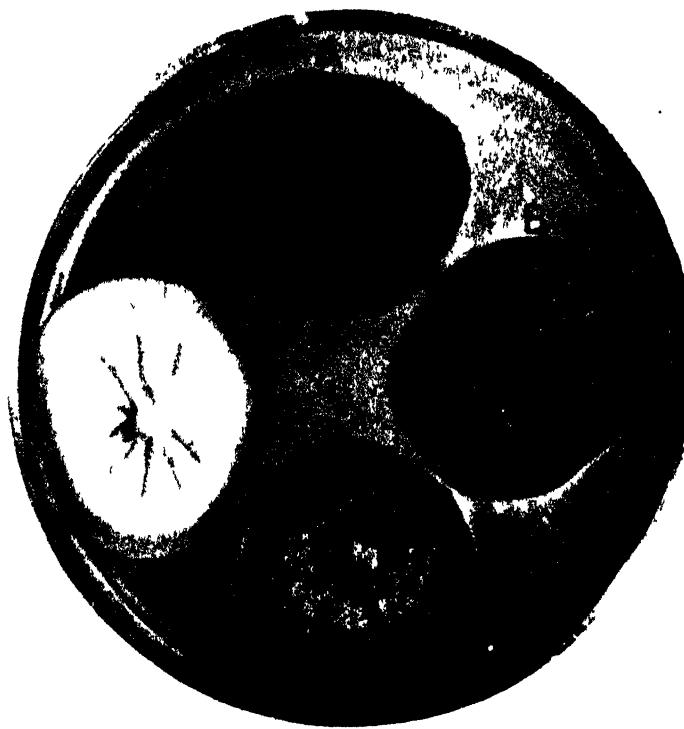


FIG. 13. Variant substrains isolated from a week-old shaken-flask culture of NRRI 1951 B25.

interest, since they reverse completely the condition existing in the Fleming strains where yields in surface culture are high, while yields in submerged culture are quite low (39).

Substrain *B* is close-textured, velvety, heavy-sporing, deep blue-green in color, and produces abundant amber exudate. Culturally it differs materially from both 1951 and 1951.B25 and more nearly resembles such substrains as 1951.B4 and 1951.B10 cited in Figures 10 and 11. Fruiting structures are generally smaller than in 1951 but do not in any sense appear atypical as they do in 1951.B25. Penicillin yields are good in both surface and submerged culture.

Substrain *C* is rather loose-textured and somewhat flocculent, but otherwise closely resembles the parent strain 1951.B25. Penicillin yields are good in both surface and submerged culture.

Substrain *D* is closely felted, very light-sporing, pale cream-buff in color and produces no exudate. Only fair yields of penicillin are produced in both surface and submerged culture.

In discussing substrains of 1951.B25 that have been tested for penicillin production, brief mention should be made of certain mutant strains which Dr. Beadle and associates have obtained from conidia irradiated with ultraviolet light. Some of these have been repeatedly tested in this laboratory in flask

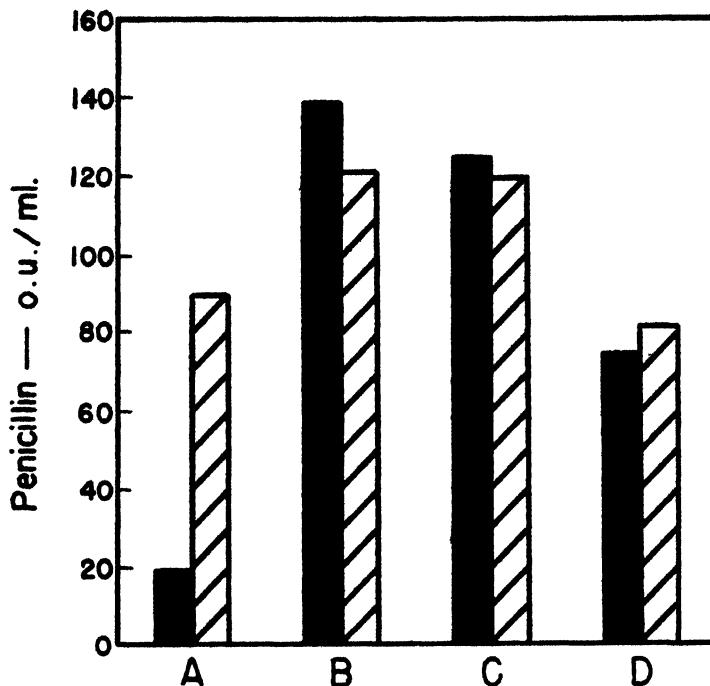


FIG. 14. Comparative production of penicillin in surface culture (solid columns) and submerged culture (broken columns) by substrains of 1951.B25, illustrated in Fig. 13. Values plotted represent average maximum yields for duplicate flasks.

cultures parallel with the stock strain of 1951.B25. In some experiments, yields from these strains have ranged from 10 to 20 per cent higher than those from the parent stock; in other experiments yields from the parent and the mutant cultures have been approximately the same. In preliminary tests with larger equipment, conducted by Dr. G. E. Ward of this Laboratory, some of these strains have produced higher yields of penicillin than the parent, NRRL 1951.B25, when tested under similar conditions. Further tests will be required to establish whether or not the mutants in question are consistently better producers. The ultraviolet-induced mutations, like the parent 1951.B25, are unstable in culture and tend to produce variant types.

This discussion of variation in the progeny of 1951 has centered around 1951.B25, and there is no necessity of introducing a further multiplicity of strains and types. It should be noted, however, that many other isolations made from 1951 and from 1951.B and 1951.C have been investigated. In general, the picture is the same for all. None are stable in culture, and from any of them markedly different substrains can be obtained by repeated cultivation and the isolation of variant sectors and colonies as they appear. From heavily sporing strains, non-sporing substrains have been obtained. And, from such very light-sporing strains as 1951.B15, illustrated in Figure 9, F, heavy-sporing substrains, approaching the original 1951, have been recovered. Yields of penicillin vary greatly, but in the majority of cases highest yields of penicillin are produced by substrains which closely resemble 1951.B25 in cultural and microscopic appearance.

#### DISCUSSION

Our objective has been to find better penicillin-producing molds, and in this quest a very large number of strains and substrains have been tested under constant conditions as a means of evaluating their relative capacities to produce penicillin. We realized that conditions and nutrient combinations which favored the production of maximum yields in a particular strain would not in all probability be optimum for other strains, particularly when these were of entirely different lineage. However, it was our belief that methods and substrates which had been found to favor the production of high yields of penicillin in known good cultures such as NRRL 1249 and 832 might in all probability reveal the identity of other good producing strains when and if such were encountered. Once such strains were discovered, optimum conditions for maximum penicillin production could be subsequently worked out. The merit of this approach has been established: (1) More productive strains for both surface and submerged production have been found; and (2), working with other apparatus and nutrient media of different composition, Dr. G. E. Ward of the Northern Regional Research Laboratory has succeeded in obtaining yields in excess of 200 O.u./ml. in submerged culture with NRRL 1951.B25, whereas the highest yield we have ever obtained in a shaken-flask culture with this strain was 168 O.u./ml.

Moyer and Coghill (34, 35) and other investigators have shown that yields of penicillin with selected strains vary with the composition of the nutrient solution, the temperature of incubation, and other factors influencing the growth and metabolism of the culture.

In surface production tests, yields of penicillin per milliliter vary substantially, depending upon the type of vessel employed, the depth of the culture solution, and the rate at which a complete covering mat of mycelium develops. In submerged tests, yields vary with differences in aeration and agitation, and in the amount and type of antifoam agent employed. In both types of culture, differences in yield may be expected when different batches of corn steeping liquor are employed. It was to minimize the various influencing factors insofar as possible that we decided to test all strains under as nearly uniform conditions

as possible. In this entire study only two batches of corn steeping liquor have been used, and in shifting from the first (a concentrated liquor containing 55 per cent solids) to the second (a spray-dried product in powder form) care was taken to adjust the concentration of the latter to a level which gave penicillin yields comparable to those previously obtained with selected test strains. It should be emphasized that there are still other factors, of unknown nature, which obviously influence the production of penicillin, particularly in submerged culture. Strains that consistently produce good yields in this laboratory sometimes fail to produce equal yields when tested in other laboratories under conditions believed to be the same. Likewise, strains that appear superior in flasks may not always produce the same yields in larger equipment. Despite the variables and imponderables inherent in the procedure, testing in flask culture constitutes the most feasible means of surveying and evaluating a large number of strains, and it is a matter of record that all high-yielding strains that are now used commercially were discovered, and their superior qualities were first verified, in small-flask cultures.

In the present study, as in the earlier survey of natural isolates (39), it has been found that appreciable variation in yield occurs in different experiments where the same strains are tested under as nearly constant conditions as it is possible to attain. For this reason, control cultures of known productivity, as measured by several successive tests, should be included. Comparisons in yield should be made with the controls of the same experiment, and no isolate should be considered exceptional upon the basis of a single test.

Molds that produce penicillin have been found to be extremely variable. In a study of 241 molds belonging to the *P. notatum-chrysogenum* group, including 189 new isolates from nature, Raper, Alexander, and Coghill (39) found penicillin production to vary from zero to levels approaching those of NRRL 1249.B21 in surface culture, and from zero to levels appreciably higher than those of NRRL 832 in submerged culture. Different levels of penicillin production could, in general, be correlated with particular cultural aspects. In the present investigation it has been found that great variability characterizes the best penicillin-producing strains, and it is believed significant that the same general pattern of variability can be observed in the progeny of entirely different stocks.

There is a marked tendency in the Fleming strain and its derivatives to produce lighter-sporing variants. Up to a certain point, these grow more rapidly, produce less exudate, and are characterized by increased penicillin production. Maximum penicillin yields are attained in cultures possessing characteristics typical of strain NRRL 1249.B21 (q.v.). Beyond this point, penicillin yields decrease with further reductions in sporulation. In extreme cases, colonies may be restricted in growth and practically non-sporulating, or rapidly growing and non-sporulating, as in the case of Hansen and Snyder's "M" type (23). None of the derivatives of the Fleming strain have shown improvement for submerged production.

From strain NRRL 1950, Dr. Beadle and associates have produced, by ultraviolet radiation, a mutation that shows marked improvement in penicillin production in surface culture. This is lighter-sporing and produces less exudate

than the parent. From this higher-yielding strain, variant types have been isolated, and one of these is characterized by flocculent, practically non-sporing colonies. Penicillin yields are much reduced. In this case, behavior parallels that of the Fleming strain, there being an increase in penicillin production with limited reduction in sporulation, followed by a marked reduction in penicillin yields as spore production approaches zero.

Strain 1951 is characterized by the greatest variability of all. The loose-textured, heavy-sporing parent produces only fair yields of penicillin in either surface or submerged culture. Closer-textured and lighter-sporing substrains, such as 1951.B25, produce greatly increased yields. On the other hand, strains that are characterized by very light sporulation consistently produce lower penicillin yields than those showing moderate spore production, although these yields are relatively better than those from poorly sporulating cultures of strain 1249.B21 or 1978.B. A strict correlation with the Fleming strains, or those derived from NRRL 1950, cannot be drawn, but similarities in behavior are suggested. As with these cultures, among the progeny of NRRL 1951 maximum yields are produced by strains showing only moderate spore production. Heavily sporing variants have been reisolated from both medium- and light-sporing strains, and these generally show somewhat reduced yields in submerged culture and fair or poor yields in surface culture. Baker (2) has shown that in *P. notatum* (NRRL 1209) anastomosis regularly occurs, followed by nuclear migrations. A heterokaryotic mycelium results, and a logical explanation is thus provided for much of the variation encountered.

Cultural changes are not always accompanied by altered capacities to produce penicillin. This is clearly seen in mutations characterized primarily by a loss or change of conidial color. An albino strain of NRRL 1951 produced through ultraviolet irradiation by Dr. Beadle and associates produces approximately the same yields of penicillin as the blue-green parent when tested in surface or submerged culture. Similarly, a naturally occurring mutant of 1951.B25 characterized by conidia of light tan color produces yields equal to the parent in both surface and submerged tests.

It is easy to visualize how yields might vary from one experiment to another in production cultures, either surface or submerged, containing elements capable of developing into such divergent cultural types as some of those illustrated above for strains NRRL 1249.B21, 1951, and others. The potency of the broth could easily be influenced if a particular type of variant gains an ascendancy in a culture vessel. This constant danger exists, but it does not assume serious proportions if stock cultures and other strains under investigation are properly maintained. Whether or not this is being done can best be judged by the agreement obtained in assays of replicate flasks in the same experiment, and in successive trials with the same organism over a period of several weeks.

A primary purpose of the present paper is to caution rather than frighten the mycologist, or microbiologist, responsible for maintaining cultures of penicillin-producing molds. These molds are characterized by unusual variability, and in general the more productive strains are the least stable. Inherent in this

variability are both advantages and disadvantages. Up to a certain level, it offers the opportunity to select more productive strains. At the same time it poses the constant and very real possibility that the desired strain may become supplanted by a less productive variant. It is not particularly difficult to maintain a selected strain in a highly productive state if certain precautions are observed. Stocks can be preserved satisfactorily in either of three forms: (1) as agar slants, (2) in dry soil, and (3) in lyophil, or desiccated, form.

Stocks maintained in agar culture should be transferred infrequently and should be recultivated upon Czapek solution agar, or some other substratum comparatively poor in nutrients. The inoculum should consist of large numbers of conidia, and it should be representative of the main body of the parent culture. Subcultures should be incubated for two weeks at a temperature most favorable for sporulation (approximately 24°-26°C. for most forms) and then stored in a refrigerator at 2°-4°C. until needed. Perpetuation of stocks by recultivation upon sporulation media is not recommended.

To preserve stocks in soil, a heavy suspension of conidia is prepared and added to carefully sterilized soil. Various types of soil, and even sand, may be employed. The soil is dispensed in tubes of convenient size, stoppered with cotton plugs, and sterilized in an autoclave overnight at 15 lbs. steam pressure. Enough spore suspension is added to each tube to moisten the soil; the tubes are allowed to dry out at room temperature, and are then stored in a refrigerator at 2°-4°C. If properly prepared and carefully handled, a single tube of this type can be used over a period of several weeks, or months, to seed sporulation media.

While either of the preceding methods can be employed satisfactorily, it is our belief that lyophil preservation constitutes the best method of maintaining valuable stocks. Conidia *en masse* are removed from a selected tube or plate culture, suspended in sterile blood serum, dispensed into small tubes, quickly frozen, vacuum-dried, and sealed under vacuum. (See Raper and Alexander (40) for a detailed discussion of this method as it has been applied to the preservation of molds.) Preparations of this type are made up in large numbers, and individual tubes are opened as often as new stocks are necessary. Using this method of preservation, one may start new cultures with conidia actually taken from the culture selected for preservation. If the original suspension is representative, there should be no doubt that the cultures resulting directly from it will be of the desired character.

The present paper is based upon a painstaking study of cultures which are today being widely used for the production of penicillin. Procedures that have led to the selection of the better-producing strains have been discussed, and the characteristics of selected high penicillin-producing strains have been presented. As work on the production of penicillin continues, it is entirely probable that more productive strains will be found either by direct isolation from natural sources or by further improvement of stocks already isolated.<sup>6</sup> It is hoped that this report will aid in this important quest.

<sup>6</sup> Since this paper was written (fall of 1944), an X-ray induced mutation of *Penicillium chrysogenum* NRRL 1951.B25 has been discovered which produces unusually high yields of

## SUMMARY

A detailed study of natural variation of molds in relation to penicillin production has been made with four selected, high-yielding cultures.

Variants of the Fleming culture of *P. notatum* Westling characterized by progressively reduced spore production and decreased exudate formation have been isolated. Up to a certain level, represented by such strains as NRRL 1249.B21, reduced sporulation is accompanied by an increased capacity to produce penicillin in surface culture. Beyond this level of sporulation, penicillin production drops off as spore formation is further reduced. White, non-sporulating strains are characterized by low yields.

*Penicillium notatum*, NRRL 832, used for the production of penicillin in submerged culture, is relatively stable. Variants have been isolated which differ appreciably in cultural aspect. None have been found to be outstandingly better producers than the parent stock, but some less productive variants have been isolated.

The stock culture of *Penicillium notatum*, NRRL 1950, is quite stable in ordinary laboratory culture, but an ultraviolet-induced mutation of this strain has been produced by Dr. G. W. Beadle and associates at Stanford University which is capable of producing substantially increased penicillin yields in surface culture. This latter strain is characterized by somewhat reduced spore production and limited exudate formation. As with the Fleming culture, very light-sporing variants produce low yields of penicillin.

*Penicillium chrysogenum*, NRRL 1951, is most unstable, and substrains capable of producing substantially increased yields of penicillin in surface and submerged culture have been isolated. NRRL 1951.B25, representative of the most productive type, is characterized by colonies of altered appearance, reduced spore production, and atypical spore-bearing structures. Very light-sporing substrains show somewhat reduced penicillin yields.

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penicillin in submerged culture. The mutant was produced by Dr. M. Demerec and associates at the Carnegie Institution, Cold Spring Harbor, Long Island, New York, and was included among a large number of isolates forwarded to the University of Minnesota where it was tested by Professor C. M. Christensen and associates under the designation X-1612. Regarding these tests, Professor Christensen writes as follows "We tested . . . about 2000 isolates from NRRL 1951.B25 which had been exposed to X-rays by Dr. Demerec. X-1612 averaged 150 Oxford units per ml. in the first two shake flask tests in November, and was sent to the University of Wisconsin at that time." Tested at the University of Wisconsin by Professors W. H. Peterson and M. J. Johnson, and subsequently by the Fermentation Division at the Northern Regional Research Laboratory, strain X-1612 has produced substantially higher yields than any other strain. In plate cultures, the mutant resembles the parent strain, NRRL 1951.B25, quite closely, both upon Czapek's solution agar and Czapek's solution agar to which 1 per cent corn steeping liquor is added (see Fig. 9E). Microscopically the strain likewise resembles 1951.B25 and shows conidial structures ranging from fairly typical for *P. chrysogenum* to structures of such bizarre patterns as illustrated in Fig. 12B.

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Methods for maintaining stock cultures at maximum levels of penicillin productivity are presented.

#### ACKNOWLEDGMENTS

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# NUTRITIONAL STUDIES OF REPRESENTATIVES OF FIVE GENERA IN THE SAPROLEGNIACEAE

BY ALMA J. WHIFFEN\*

*Research Laboratories, The Upjohn Company, Kalamazoo, Michigan*

Members of the Saprolegniaceae, a family of water molds, have been grown and studied on a variety of substrata, which include larvae and cadavers of insects; various seeds; albumen of egg; haemoglobin; decoctions of corn meal, beans, and peas; and mixtures of peptone and crude maltose. Growth experiments, using these complex nutrilites, were performed by Klebs (1899), Pieters (1915), and Coker (1923). In 1932 Volkonsky, working at the Pasteur Institute succeeded in growing an undetermined species of *Saprolegnia* in a culture medium that was completely defined chemically. Volkonsky's work was followed by that of Moreau and Moreau (1940). It is the purpose of this paper to extend our knowledge of the nutrition of the Saprolegniaceae by presenting comparative nutritional studies of five commonly-occurring genera, *Saprolegnia*, *Achlya*, *Thraustotheca*, *Dictyuchus*, and *Aphanomyces*. For morphological and taxonomic discussions of these genera the reader is referred to the monograph on the Saprolegniaceae by Coker (1923).

## EXPERIMENTAL PROCEDURE

Samples of soil were covered with water and baited with halves of boiled hemp seeds (*Cannabis sativa*). Of the species of water molds thus obtained five were selected for nutritional studies: *Saprolegnia ferax*, *Achlya flagellata*, *Thraustotheca clavata*, *Dictyuchus monosporus*, and *Aphanomyces stellatus*. Pure cultures of these species were maintained on agar slants of 0.1% of Bactopeptone and 0.5% of Difco Malt Extract.

A mineral salt solution, containing 0.002 M  $MgSO_4 \cdot H_2O$ , 0.001 M  $CaCl_2$ , 0.000002 M  $FeSO_4$ , 0.00001 M  $ZnSO_4 \cdot 7H_2O$ , and  $K_2HPO_4$  plus  $KH_2PO_4$  to equal a  $PO_4$  concentration of 0.005 M and a pH of 6.0, was empirically adopted as the mineral salt base in all media. All cultures were incubated at 26° C.

Very adequate growth of all five species was obtained when 0.2% of Bactopeptone and 0.05% of glucose were added to the mineral salt base. It was possible, however, that the glucose was not being utilized and that the peptone was serving as the source of carbon and energy as well as of nitrogen in this medium. To test this hypothesis, two series of liquid media were prepared, one containing only peptone in concentrations of 1.0%, 0.5%, 0.1%, 0.05%, and 0.01% and one with 0.5% of glucose added to each concentration of peptone. Table I summarizes the results of this experiment.

It was probable that casein, like peptone, would serve as an excellent source of nitrogen; but when the growth of the five species in 0.2% of casein and 0.5% of glucose was compared with that obtained in 0.2% of peptone and 0.5% of glucose, it was found that the growth in the peptone was noticeably superior to that in

\* See page 308. -

casein. Comparison of growth in the casein-glucose medium with that in the casein medium without glucose gave assurance that the glucose in the casein-glucose medium was being utilized. It was suggested that growth substances in the peptone might be responsible for the enhanced growth in peptone. If the peptone and to a more limited extent the casein were furnishing these organisms with one or more vitamins that they were unable to synthesize, one would expect to find none or very little growth in a vitamin-free medium. Therefore, hyphae of each species, grown in casein-glucose medium, were washed in distilled water and transferred to 500 ml. Erlenmeyer flasks, each containing 50 mls. of a medium with 0.2% of vitamin-free casein (Pfanstiehl) and 0.5% of glucose. After three days of incubation, hyphae were inoculated into fresh vitamin-free casein-glucose medium. No diminution in growth resulted after these two transfers in vitamin-free media.

TABLE I

*The effect of five concentrations of peptone with and without 0.5% of glucose upon growth and reproduction of five species of water molds*

CONCENTRATION OF PEPTONE	SAPROLEGNIAS	ACHLYA	THRAUSTOTHECA	DICTYUCHUS	APHANOMYCES
1.0%	+++	+++	+++	+++	+++
0.5%	++	++	++	++	++
0.1%	+	+	+	+Z	+
0.05%	+Z	+O	+Z	+Z	+
0.01%	+Z	+OZ	+OZ	+Z	+
1.0%—glucose	+++++	+++++	+++++	+++++	+++++
0.5%—glucose	++++	++++	++++	++++	++++
0.1%—glucose	+++	++O	++	++	++
0.05%—glucose	++O	++O	++OZ	++	++
0.01%—glucose	++O	++O	++OZ	++	++

+++++ = excellent growth, ++++ = good growth, +++ = moderate growth, ++ = slight growth, + = very slight growth. Z = zoosporangia produced, O = oogonia produced.

The experiment just described, however, did not obviate the possibility that the addition of one or more vitamins to the vitamin-free casein medium would increase the amount of growth significantly. Nine synthetic vitamins, riboflavin, calcium pantothenate, nicotinic acid, pyridoxin, inositol, thiamine, choline, para-amino-benzoic acid, and  $\beta$ iotin (SMACO crystalline), were tested for their ability to stimulate the growth of the five species. Each of duplicate 500 ml. Erlenmeyer flasks, containing 50 mls. of 0.05% of vitamin-free casein and 0.5% of glucose, received a single supplement of one of the vitamins in a concentration of 20  $\mu$ g. per flask, with the exception of biotin, of which 2.5  $\mu$ g. were used. The flasks were inoculated with hyphae from cultures of each species in vitamin-free casein and glucose. At the end of four days of incubation, the dry weight of the mycelium from each flask was determined. The dry weights of the mycelium from duplicate flasks agreed well and were averaged. The

results are compiled in Table II. It was apparent that no significant increase in the dry weight of any one of the species was caused by the addition of any one vitamin to the culture medium.

In order to discover whether these water molds were able to utilize inorganic nitrogen compounds and amino acids as sources of nitrogen, culture media were prepared with 0.5% of glucose and 0.05% of one of the following compounds:  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{NaNO}_3$ , glycine, alanine or glutamic acid. Glycine and alanine were chosen because Volkonsky (1932) had reported growth of his species of *Saprolegnia* with these two amino acids. Glutamic acid was tested as a possible favorable N-source because it constitutes approximately 21% of casein. Inoculations of these culture media were made with mycelium from casein-glucose cultures of the five species. None of the species grew in the media containing inorganic nitrogen. *Saprolegnia ferax* and *Thraustotheca clavata* made only very slight growth in the glycine medium and the other three species no growth at all.

TABLE II

*The effect of single vitamins upon the growth of five species of water molds*  
Dry weight of mycelium expressed in milligrams

VITAMIN SUPPLEMENT	SAPROLEGINIA	ACLYA	THRAUSTOTHECA	DICTYUCHUS	APHANOMYCIS
Riboflavin	15.4	23.3	19.5	19.9	15.3
Ca pantothenate	15.6	20.1	20.3	19.4	14.0
Nicotinic acid	16.7	20.8	23.9	17.2	11.4
Pyridoxin	20.3	17.6	19.1	27.7	17.1
Inositol	14.7	21.6	23.2	15.3	13.3
Thiamine	16.6	17.4	23.3	15.3	17.2
Choline	19.6	21.1	23.3	15.8	15.2
Para-amino-benzoic acid	16.1	22.2	23.5	23.3	14.8
Biotin (SMACO cryst)	20.0	22.7	20.2	26.4	14.4
Control (no vitamin supplement)	27.0	20.8	20.7	17.5	16.5

All five species grew well in the media containing alanine and glutamic acid but the amount of growth did not equal that obtained in the casein medium.

At this point it became possible to determine whether these five species of water molds resemble Volkonsky's species of *Saprolegnia* (Volkonsky, 1932-1934) in requiring a form of organic sulphur for growth. Since both casein and peptone contain cystine, the inferiority of the growth in glutamic acid to that in casein and peptone could be explained on the basis of the inability of these water molds to reduce the sulphate supplied in the mineral salt solution. After three successive transfers in a liquid medium containing 0.05% of glutamic acid and 0.5% of glucose and no organic sulphur, there was a slight diminution in growth and the amount of growth was markedly less than that in the casein medium used as a control for this experiment. The fourth transfer was made into flasks of glutamic acid-glucose medium without cystine and with cystine in a concentration of 0.005%. By the end of three days, growth in the flasks containing

cystine was approximately three times greater than that in the flasks lacking cystine. Furthermore, the growth obtained in the medium of glutamic acid, glucose, and cystine was superior to that in the casein-glucose medium. The requirement of organic sulphur by these five members of the Saprolegniaceae is confirmation of Volkonsky's initial discovery.

It must be noted that a definite amount of growth was made in the glutamic acid-glucose medium which lacked cystine. It can be assumed that these water molds were able to reduce sulphate to a limited extent and that some sulphur might have been obtained by autolysis of the inoculum. Substitution of chlorides for sulphates in the mineral salt solution would solve this problem but such an experiment was not performed.

In order to determine the concentration of cystine that is the most favorable to growth, the five species were inoculated into flasks of glutamic acid-glucose

TABLE III

*The effect of six sulphur-containing compounds and five different concentrations of cystine upon growth of five species of water molds*

SULPHUR COMPOUND	SAPROLEGNIA	ACHYLYA	THRAUS-TOTHECA	DICTY-UCHUS	APHAN-OMYCES
Thiamine	+	+	+	+	+
Glutathione	++++	++++	++++	++++	++++
Cysteine	++++	++++	++++	++++	++++
Thiourea	+	+	+	+	+
Na thioglycollate	+	+	+	+	+
dl-methionine	++++	++++	++++	++++	++++
Cystine 0.003 M	++++	++++	++++	++++	++++
Cystine 0.001 M	++++	++++	++++	++++	++++
Cystine 0.0001 M	+++	+++	+++	+++	+++
Cystine 0.00001 M	+++	+++	+++	+++	+++
Cystine 0.000001 M	+	+	+	+	+

++++ = good growth, +++ = moderate growth, + = poor growth.

medium, supplemented by one of the following concentrations of cystine: 0.003 M, 0.001 M, 0.0001 M,  $1 \times 10^{-5}$  M, or  $1 \times 10^{-6}$  M. Inoculations were made with hyphae from a glutamic acid-glucose medium that lacked cystine. The results of this experiment are included in Table III. Only two concentrations of cystine, 0.003 M and 0.001 M, were adequate for the support of the maximal amount of growth of all five species.

It was of interest to test a few other sulphur-containing compounds as S-sources for the water molds being studied. Six compounds were chosen to be used as single supplements to the glutamic acid-glucose medium and were employed in the following concentrations: thiamine 0.0001 M, glutathione 0.0001 M, cysteine hydrochloride 0.001 M (Seitz-filtered), thiourea 0.001 M, sodium thioglycollate 0.001 M, and dl-methionine 0.001 M. Thiamine, thiourea, and sodium thioglycollate were not utilized as a source of sulphur but glutathione, cysteine, and methionine were equal to cystine as S-sources (see Table III).

Of the compounds which Volkonsky (1933) tested as S-sources for his species of *Saprolegnia*, cystine and cysteine were the best and hydrogen sulphide, thioacetic acid, thiocarbonic acid, and thiosulphuric acid were of only mediocre value.

After the demonstration of the need of these water molds for sulphur in a reduced form, it was possible to proceed with the testing of compounds as sources of nitrogen. Alanine, glycine,  $(\text{NH}_4)_2\text{SO}_4$ , and  $\text{NaNO}_3$  were retested with cystine added to the medium. In addition to these four compounds, urea, asparagine, and seventeen amino acids were studied, each in a concentration of

TABLE IV

*The availability of nitrogen-containing compounds for growth of five species of water molds*

0.05% OF N-COMPOUND, 0.5% OF GLUCOSE, AND 0.001 M CYSTINE	SAPROLEGNIA	ACELIA	THRAUS- TOTRECA	DICTY- UCHUS	APHAN- OMYCES
Peptone	++++	++++	++++	++++	++++
Casein	++++	++++	++++	++++	++++
Urea	+	+	+	0	0
Asparagine	++++	++++	++++	+++	++++
Glycine	++	0	++	0	0
d-alanine	++++	++++	++++	++++	++++
d-valine	+++	++	+++	++++	+++
l-leucine	+++	++	++	+++	+
l-phenylalanine	+++	+++	+++	++++	+
l-tyrosine	++++	++++	++++	+	++++
d-lysine	++	+	++	+++	+
d-arginine	++++	++++	++++	++++	+++
d-histidine	+++	+++	+++	0	++++
d-glutamic acid	++++	++++	++++	++++	++++
d-aspartic acid	+++	+++	+++	+++	+++
l-proline	++++	++++	++++	++++	++++
l-hydroxyproline	0	+	0	+	++
l-serine	+++	+	++	++	++
l-threonine	++	++	++	+	0
l-cysteine	0	0	0	0	0
l-methionine	++	+++	++	+++	0
$(\text{NH}_4)_2\text{SO}_4$	0	0	0	+	0
$\text{NaNO}_3$	0	0	0	+	0

++++ = good growth, +++ = moderate growth, ++ = slight growth, + = very poor growth, 0 = no growth

0.05% with 0.5% of glucose and 0.001 M cystine. The inoculum was grown in a liquid glutamic acid-glucose-cystine medium, from which a small number of hyphae of each species was transferred to 250 ml. Erlenmeyer flasks containing 20 ml. aliquots of the media to be studied. The cultures were incubated for three days. Casein-glucose and peptone-glucose media were used as controls. The results of this experiment are condensed in Table IV. The nitrogen in  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NaNO}_3$  was unavailable for growth to all species except *Dictyuchus monosporus*, which made only a very small amount of growth in these two media. The growth of *Saprolegnia* and *Thraustotheca* in glycine was only

slightly improved by the addition of cystine to the medium. Threonine and methionine were utilized by all species except *Aphanomyces stellatus*. Urea supported very poor growth of *Saprolegnia*, *Achlya*, and *Thraustotheca* and none at all of *Dictyuchus* and *Aphanomyces*. Good growth was attained in histidine by all species except *Dictyuchus monosporus*, which did not grow at all in this medium. Not one of the five species grew in cysteine but this failure may be attributed to the lowering of the oxidation-reduction potential of the medium by the cysteine. A very limited amount of growth, however, was made in a medium containing glucose and 0.01% of cysteine. Glutamic acid, proline, and alanine were the most favorable nitrogen sources for all five species. *Saprolegnia ferax* and *Thraustotheca clavata* were similar in their utilization of nitrogen compounds.

The inability of an inorganic nitrogen compound to support the growth of these water molds pointed to the necessity of using peptone or an amino acid as the nitrogen source in testing a series of compounds as sources of carbon and energy for growth. It had already been shown that 0.05% of peptone is able to support a significant amount of growth. Likewise, the single amino acids, glutamic acid and alanine, in a concentration of 0.05% are able to support a moderate amount of growth. It was then apparent that the compounds most favorable as N-sources were also capable of providing the carbon and energy required for growth and would interfere with a clear-cut determination of the utilization of carbon compounds present in the same medium. However, in earlier studies it was shown that the addition of 0.5% of glucose, a readily utilizable carbon source, to 0.05% of peptone resulted in a great increase in the quantity of growth. This increase in the density of growth was particularly marked in a peptone-glucose medium solidified with 2% of agar. Therefore, the method adopted for judging the utilization of a carbon compound consisted of comparing visually the density of growth of the species on an agar plate of 0.05% of peptone with the density of growth of that species on agar containing the same concentration of peptone and 0.5% of the carbon compound. The results were not unequivocal but were judged to be satisfactory for the purpose at hand.

A total of eighteen compounds were studied as sources of carbon and energy. The agar plate was inoculated in the center by a disc of agar, 4 mm. in diameter, cut from the margin of a three-day-old colony of the water mold on maltose-peptone agar. Plates were inoculated in duplicate and the density of growth was judged after three days of incubation. Sodium acetate and sodium citrate in a concentration of 0.5% were extremely toxic; in a concentration of 0.01% these two compounds were neither toxic nor effective in increasing growth. Glucose was the only carbon source utilized by all species; in fact, it was the only carbon compound available to *Dictyuchus monosporus*. Sucrose was utilized by only *Thraustotheca clavata* and *Achlya flagellata*. Of the sugars and alcohols tested, xylose, galactose, lactose, and mannitol were unavailable to all species. Maltose, starch, and glycogen were good carbon sources for all species except *Dictyuchus monosporus* (see Table V).

Data regarding the phosphate tolerance of an organism are useful in any

experiments requiring the use of a phosphate buffer. The degree of phosphate tolerance of the five species was determined by inoculating aliquots of glutamic acid-glucose-cystine medium, buffered with phosphate in concentrations of 0.1 M, 0.01 M, 0.005 M, and 0.001 M, and observing the effect of the phosphate concentration upon the growth of each species (see Table VI). Growth was inhibited in 0.1 M phosphate and slightly inhibited in 0.01 M phosphate. A concentration of 0.005 M phosphate was the most favorable to growth.

To determine the limits of pH within which growth of the water molds would take place, the pH of 20 ml. aliquots of glutamic acid-glucose-cystine medium

TABLE V

*The availability of carbon-containing compounds for growth of five species of water molds*

0.5% OF C COMPOUND PLUS 0.05% OF PEPTONE	SAPROLEGNIA	ACHLYA	THRAUSTOTHECA	DICTYUCHUS	APHANOMYCES
d-arabinose	0	0	0	0	+
d-xylose	0	0	0	0	0
l-rhamnose	0	+	0	0	+
d-glucose	++++	++++	++++	++++	++++
d-mannose	+	+	0	0	0
d-galactose	0	0	0	0	0
d-fructose	+	+	0	0	+
Maltose	++++	++++	++++	0	++++
Lactose	0	0	0	0	0
Sucrose	0	++++	++++	0	0
Raffinose	0	+	0	0	0
Dextrin	+++	+++	++	0	++
Soluble Starch	++++	++++	++++	0	++++
Glycogen	++++	++++	+++	0	+++
Mannitol	0	0	0	0	0
Glycerol	+	+	0	0	+
Na citrate 0.01%	0	0	0	0	0
Na acetate 0.01%	0	0	0	0	0

++++ = growth very much better than on 0.05% of peptone, +++ = growth much better than on 0.05% of peptone, ++ = growth moderately better than on 0.05% of peptone, + = growth slightly better than on 0.05% of peptone, 0 = growth not better than 0.05% of peptone.

in 250 ml. Erlenmeyer flasks was adjusted to give a pH range from 2.1 to 9.0 previous to the inoculation of the flask with one of the five species of water molds. The results are shown in Table VI. No growth occurred at pH 2.0, 3.0, and 9.0. Growth at pH 6.9 and 7.8 was strongly inhibited. Excellent growth was obtained at pH 4.2, 5.0, and 5.8. Measurement of the pH in each flask after three days of incubation showed a decrease in acidity.

All attempts to correlate the production of reproductive structures with the qualitative character of the nutrient medium were unsuccessful. Under no conditions of culture, except on hemp seed and grass leaves, did *Aphanomyces stellatus* produce zoosporangia and oogonia. Zoosporangia were formed by

*Thraustotheca clavata* in all carbon compounds tested and by *Dictyuchus monosporus* in all but glucose of the carbon series. In addition *Thraustotheca clavata* formed oogonia in lysine, urea, proline, asparagine, casein and peptone of the series of nitrogen compounds. Oogonia of *Achlya flagellata* were produced in urea, lysine, arginine, proline, alanine, tyrosine, and asparagine in the nitrogen series and in all of the carbon compounds except mannose and galactose. Zoosporangia of *Achlya flagellata* and *Saprolegnia ferax* appeared in 0.01% and 0.05% of peptone. Oogonia of *Saprolegnia ferax* appeared in solutions containing glucose, glycogen, urea, and arginine.

The type of nutrient influenced the size of the oogonia and eggs and was a factor in determining the percentage of oogonia which matured and the number

TABLE VI

*The effect of variation in phosphate concentration and pH upon the growth of five species of water molds*

PO <sub>4</sub> CONCENTRATION AND INITIAL pH	SAPROLEGINIA	ACHLYA	THRAUSTOTHECA	DICTYUCHUS	APHANOMYCES
pH 6.0					
PO <sub>4</sub> 0.1 M	+	+	+	+	+
PO <sub>4</sub> 0.01 M	++	++	++	++	++
PO <sub>4</sub> 0.005 M	++++	++++	++++	++++	++++
PO <sub>4</sub> 0.001 M	+++	+++	+++	+++	+++
PO <sub>4</sub> 0.005 M	final pH	final pH	final pH	final pH	final pH
pH 2.1	0	0	0	0	0
pH 3.0	0	0	0	0	0
pH 4.2	++++ 6.6	++++ 6.5	++++ 6.4	++++ 6.0	++++ 6.4
pH 5.0	++++ 7.0	++++ 7.0	++++ 6.9	++++ 6.4	++++ 7.0
pH 5.8	++++ 7.0	++++ 6.9	++++ 7.0	++++ 6.4	++++ 7.0
pH 6.9	+	+	+	+	+
pH 7.8	+	+	+	+	+
pH 9.0	0	0	0	0	0

++++ = excellent growth, +++ = good growth, ++ = moderate growth, + = slight growth, 0 = no growth.

of antheridia that were formed. This fact was particularly applicable to *Achlya flagellata*. In general, however, the postulate of Klebs was true—that a high concentration of nutrient, which favored vegetative growth, was unfavorable to the formation of oogonia and zoosporangia. This fact was well demonstrated in the experiment in which a series of peptone concentrations with and without glucose were studied (see Table I).

#### DISCUSSION

Volkonsky has made a more detailed study of the nutrition of *Saprolegnia* than any previous worker. He found that his species of *Saprolegnia* utilized glucose, maltose, and dextrin but not mannitol, fructose, sucrose, galactose, and lactose. With the exception of fructose his results agree with those reported in this paper for the utilization of carbon compounds by *Saprolegnia ferax*. Of the

nitrogen sources which Volkonsky tested, glycine, alanine, serine, valine, leucine, cystine, arginine, lysine, aspartic acid, asparagine, glutamic acid, glucosamine, phenylalanine, tyrosine, tryptophane, proline, histidine, ammonia in organic salts, and urea were utilized but sarcosine, acetamid, triethanolamine, and potassium nitrate were not used. The availability to *Saprolegnia ferax* of cystine, glucosamine, ammonia in organic salts, sarcosine, acetamid, and triethanolamine was not determined but the utilization by *S. ferax* of the remaining N-compounds in the above list coincides with that of Volkonsky's species of *Saprolegnia*.

It is interesting to compare the nutritional requirements of *Leptomititus lacteus* and *Apodachlya brachynema*, species of the Leptomitaceae that have been studied by Schade (1940), with those of the five species of the Saprolegniaceae investigated in the present study. Both species of *Leptomititus* and *Apodachlya* were able to reduce sulphate in order to satisfy their sulphur requirements. The species of *Leptomititus* and *Apodachlya* are like the species of *Saprolegnia*, *Achlya*, *Thraustotheca*, *Dictyuchus*, and *Aphanomyces* in their non-utilization of inorganic nitrogen but differ from these five species in their ability to utilize acetate and in the inability of *Leptomititus* to utilize sugars as carbon sources.

The nutrition of *Saprolegnia ferax*, *Achlya flagellata*, *Thraustotheca clavata*, *Dictyuchus monosporus*, and *Aphanomyces stellatus* is characterized by the importance of the high molecular nitrogenous compounds and amino acids as sources of both nitrogen and carbon and energy. A similar condition was found in four species of *Allomyces* by Wolf and Shoup (1943).

#### SUMMARY

A survey is made of the nutritional requirements of five species of the Saprolegniaceae, *Saprolegnia ferax*, *Achlya flagellata*, *Thraustotheca clavata*, *Dictyuchus monosporus*, and *Aphanomyces stellatus*. All five species are able to grow in a vitamin-free medium and show no increase in growth upon the addition of any one of nine vitamins to the vitamin-free medium. It is therefore assumed that these species are able to synthesize all the vitamins required for their growth. The growth requirement of sulphur in an organic form is satisfied by cysteine, cystine, glutathione, and methionine. Inorganic nitrogen is unavailable for growth but peptone, casein, and a number of amino acids are excellent sources of nitrogen. The only carbon compounds of importance in the nutrition of the five species are glucose, maltose, starch, and glycogen. Glucose is the only carbon source available to *Dictyuchus monosporus*. A pH range from 4.0 to 6.0 and a phosphate concentration of 0.005 M are optimal for growth of all five species. Observations were made on the relation between the composition of the culture medium and the production of zoosporangia and oogonia by the five species.

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# REVISION OF THE GENUS COELOMOMYCES, PARASITIC IN INSECT LARVAE

By J. N. COUCH

*Department of Botany, University of North Carolina*

## PLATES 1 AND 2

### HISTORICAL

The peculiar genus *Coelomomyces* was established in 1921 by Keilin to include a fungus found growing within the body cavity of a single larva of the yellow fever mosquito, *Stegomyia scutellaris*. The new species was named *Coelomomyces stegomyiae* Keilin. Examined under a low power, the larva's body was almost completely filled with oval bodies having a thick yellowish wall. By studying teased material and sections, fragments of a rather well developed coenocytic, irregular, anastomosing mycelium were discovered mostly around the midgut and just below the hypoderm. Keilin considered the thick-walled, oval, yellowish bodies as sporangia whose development is as follows. The majority of the branches show terminal multinucleated thickenings with irregular contours. These are separated from the threads and become free in the host's perivisceral fluid where they increase considerably in size. After a time they decrease in size due to greater density of the protoplasm, the nuclei multiply by division, they assume a sub-oval shape flattened on one side, and an even contour, and the wall begins to thicken. This becomes double-layered with a thin, hyaline, internal part and a thick, yellowish, pitted, external layer. The pits are small and lenticular and there is a fine line running from pole to pole on the convex side.

In the fixed and sectioned material, stages in zoospore formation were described and illustrated. By cleavage the protoplasm is divided into uninucleate masses which are round at first but become lozenge-shaped when mature, and  $5\mu$  long by  $1\mu$  thick. Keilin suggested that the escape of the spores probably results from the rupture of the sporangium along the clearly marked line of cleavage on the convex surface. By compressing the sporangium between the cover-slip and slide he was able to observe such a rupture. The mycelium of *Coelomomyces*, being devoid of cross walls, would appear to belong to the Phycomycetes, and undoubtedly shows some resemblance to the Chytridineae, such as *Physoderma* and *Catenaria* which have a more or less similar development of the sporangia within the tissue or body cavity of the host. The structure of the sporangia of *Coelomomyces* differs, however, in many respects from that of the Chytridineae and its mycelium appears to be better developed than is the case in any known representative of this group of fungi. Keilin's final conclusion was that the systematic position of *Coelomomyces* could not be established until more abundant and living material was studied.

A year later Bogoyavlensky (1922) described a new organism under the name of *Zografa notonectae* n.g., n. sp., which he found parasitic in the body cavity of

*Notonecta* (Hemiptera) collected in ponds in Moscow, U.S.S.R. According to the author, in the vegetative stages the fungus is composed of a plasmodium of irregular, much-branched and anastomosing threads. The plasmodium does not possess independent motion. The hyphae form terminal swellings which increase in size and become detached as oval, plurinuclear bodies, which develop into sporangia. These now decrease in size and become surrounded by a hyaline membrane which soon thickens and turns yellowish or brown. The nuclei migrate to a peripheral position. In one case the author noticed the breaking up of the protoplasm of a sporangium into a number of small, amoeboid, uninuclear bodies, but was doubtful if this was the usual behavior. He considered *Zografa* as related to *Sporomyxa scauri* Léger.

In 1927 Keilin published a brief paper showing that his species, *Coelomomyces stegomyiae*, and Bogoyavlensky's *Zografa notonectae* were so closely related that they should be included in the same genus, and since *Coelomomyces* had priority, *Zografa notonectae* became *Coelomomyces notonectae*. Keilin in this paper again called attention to the uncertain systematic position of this genus.

In 1935 Iyengar described two closely allied new species of *Coelomomyces* found parasitizing various species of *Anopheles* larvae in India, namely, *C. indiana* and *C. anophelesica*. The mycelium in both species is tubular, dichotomously branched, according to his figures, and very thin-walled, 7-14 $\mu$  thick in *C. indiana* and 7-12 $\mu$  in *C. anophelesica*, and attached to the fat body of the host apparently by very minute hyphae. In *C. indiana* the sporangia are oval, 25-36 x 38-60 $\mu$ , and with a thick (1-3 $\mu$ ), opaque, yellow wall, with 7-10 branched, longitudinal, elevated ribs. The wall between the ribs is about 1 $\mu$  thick and the drawings (figure 4 c, f) suggest striations between the ribs though there is no mention of this in the text. The author states that the wall in section is striated and one-layered. In *C. anophelesica* the sporangia are 28-40 x 34-44 $\mu$  and are asymmetrical, sub-circular in shape, with one side flat and the other strongly convex, with the thickness less than the breadth, thickness 16-23 $\mu$ . The wall has several elevated ribs running in concentric or eccentric circles, the middle ribs branched and irregular; it is 2.6-3.4 $\mu$  thick through the ribs, and 3 $\mu$  thick between the ribs. In section it has the striae as in *C. indiana*. According to the description, the inner spore wall is lacking in both species, though the figures of the young sporangia of *C. indiana* (Fig. 4, a, b) show a double-layered wall.

Iyengar was in agreement with Keilin that while the systematic position of *Coelomomyces* was still quite doubtful it should be placed tentatively with the Chytridiales.

From Sierra Leone on the coast of West Africa, Walker (1938) recorded four forms or types of *Coelomomyces* in larval and adult stages of *Anopheles costalis* and *A. funestus*. Infected larval stages were much more abundant than were infected adults. No differences were detected in the mycelia of the four types, the thickness of the threads varying from 3-8 $\mu$  in each. The four types may be distinguished from one another only after the sporangia are formed. The sporangia in types 1 and 4 measure between 45 and 60 $\mu$  in length, and in types 2 and 3 from 25-35 $\mu$ . Types one and two are oval and in the photographs show

what might be interpreted as a two-layered wall, though Walker seems in doubt on this. Type 3 is sub-oval and flattened on one side with a somewhat crenated wall. Walker does not give the thickness of the sporangia, only the length is given, but by measuring his photographs it is seen that in type 3 the sporangia are about half as thick as long, and thus  $14-18 \times 25-35\mu$ . In the photograph of type 4, figure 4, and less distinctly in type 3, figure 3, a double-layered wall is evident. For type 3, which proved to be by far the most common of the forms, Walker proposed the name *Coelomomyces africanus*, n. sp. The other three types were not given a name. Numerous attempts were made by Walker to culture all four types of sporangia and two kinds of mycelia on various artificial media, but without success.

The most interesting part of Walker's paper has to do with his attempts to produce artificial infection of the mosquitoes. Eggs, fresh larvae, and pupae were put in water containing sporangia, either intact or ground up after drying, or material from infected larvae or living infected larvae; but the results were negative. Larvae may swallow the sporangia but pass them out apparently unchanged. For other experiments, one particular breeding-place (field 2), which had consistently produced infected larvae practically throughout the year, was flooded by irrigation after it had been almost completely dry for two days. A few laboratory-bred larvae of *A. costalis* isolated in a very fine-mesh copper sieve in the above pool, and 66% of the larvae of *A. costalis* which developed naturally in the pool outside of the sieve became infected. Within a few days a small number of laboratory-bred larvae also became infected when placed in a concrete pool containing water, sediment, and vegetation transferred from the pool in field 2. Twenty-eight days after the concrete tank was filled, 1000 young larvae and about 600 eggs were put in it, but none subsequently showed any trace of infection. The concrete tank was cleaned and filled with 30 gallons of clear water from the brook, well below the point where the irrigation was led off. Large numbers of clean larvae and eggs put in the tank failed to show any infection. Walker suggests that the source of infection is present in the water but does not survive for long.

A possible interpretation of these very interesting experiments with *Coelomomyces* may be made on the basis of some of my researches (1945) on a distantly related form, *Catenaria allomycis*. In this species the resting bodies germinate only after they have been dried and then, when flooded, only a few germinate at any one time. The rest must be dried again and flooded again before they are ready to germinate. In Walker's experiments it is likely that when "field 2" was flooded, a certain number of resting sporangia present in the soil germinated. It is very likely that the resting sporangia of *Coelomomyces africanus* cannot germinate until dried. Indeed Walker kept them in water for over a year without obtaining germination.

So far none of these observers had seen sporangial germination. This very critical stage was followed by Meillon and Muspratt (1943) in an unidentified species of *Coelomomyces* found growing in a larva of a species of *Mucidus* (Culicidae) collected in Northern Rhodesia. When germinating the sporangium

begins to swell, a slight bulge appearing on one side. This enlarges and ruptures the outer hard wall along the longitudinal line mentioned by Keilin (1921) and the contents of the sporangium flow out surrounded by two thin membranes. Within a few minutes the closely packed spores come to life and begin moving within the membranes, gradually gaining in speed until the spores within the membranes and in the sporangium are a seething mass. A few seconds later the spores push through the membranes and swim away with a single posterior flagellum. The zoospore body measures about  $4\mu$ , and the length of the flagellum is approximately four times this length. It would be interesting to know if the sporangia were dried before germination.

Up until now, five species of *Coelomomyces* have been described, one from the Federated Malay States, one from Moscow, U.S.S.R., two from India, and one from West Africa. Haddow (1942) has reported *C. africanus* Walker from Kenya in East Africa on the adult females of *Anopheles funestus* and *A. gambiae*. He noticed that in every case the ovary was primarily affected, being completely disorganized and semifluid in later stages, and filled with masses of sporangia which at this stage were sometimes found in other parts of the body. Infected larvae were also found. Unidentified species of this genus have been reported by Thompson (1924) from Malay, and by Meillon and Muspratt (1943) from Northern Rhodesia.

As first indicated by Walker (1938) it is likely that the fungi described as parasitic within mosquito larvae by Eckstein (1922); Chorine and Baranoff (1929); Manalang (1930); Gibbins (1932); and Feng (1933) belong in the genus *Coelomomyces*. There is also a possibility that the fungus described by Wize (1905) as *Olpidiopsis ucrainica* found in the larvae and pupae of *Cleonus puncti-ventris* and in one pupa of *Anisophia austriaca* and recently considered the same by Sparrow (1939) as *Myiophagus Thaxter*, n. gen., found in dipterous pupae, may be closely enough related to *Coelomomyces* to include both genera in the same family.

In spite of the rather considerable attention given to these organisms by animal parasitologists, their life histories are incompletely known and their systematic position so doubtful that none of the mycological textbooks have even mentioned the genus.

It is the purpose of this paper to describe five new species of this genus and to record some observations which would seem to give rather definite clues as to the systematic position of these highly specialized parasites. This is the first time these parasites have been reported in the Western Hemisphere.

All of the forms to be described here were collected in Georgia by inspectors of the State Malaria Control in War Areas, and were sent to me by Mr. Harold R. Dodge of the same department, who furnished me with the following information. "These parasites were first noticed in the fall of 1942 in *Psorophora ciliata*, but were not seen again until May 1944. *Anopheles crucians* and *Culex erraticus* are the two species most commonly infested, but it is doubtful if parasitism is ever high enough effectively to reduce the breeding of any species.

"Infested larvae were first sent to the United States National Museum, where

Dr. Alan Stone considered the parasites to be gregarines and suggested sending them to Dr. David Wenrich. The latter, on examining developmental stages, suspected their fungal nature and suggested sending material to a mycologist.

"In view of the intensive study which has been made of mosquitoes since their relationship to human disease was discovered, it is interesting that these fungi were so long unrecognized in this country. This may be due to the close similarity with protozoan forms, which has caused them to be confused with the Sporozoa or Gregarinida.

"Infested larvae are recognizable by the spore-like bodies which are visible through the soft integument of the body. When sparse some may be overlooked, but when abundant they cause the host larva to show an unusual rusty brown color. This color is about the only way one can distinguish parasitism in *Culex erraticus* without dissection, for larvae of this species are densely pilose and the parasites small."

The first two larvae examined by me, were completely filled with brown, egg-shaped, thick-walled bodies which in size, shape, and structure suggested the resting bodies of *Allomyces*, but since there seemed to be no mycelium present, I suspected that the bodies might be cysts of protozoa or worm eggs, and had them examined by our protozoologist, Dr. C. Dale Beers, who suggested that the brown bodies did not belong to any animal with which he was familiar. Meanwhile in fresh, living material from Mr. Dodge, I had found the brown bodies germinating to form zoospores somewhat similar to those in the Blastocladiales, and further examination of living parasitized larvae showed the very inconspicuous mycelium.

These peculiar fungi, though related to the Blastocladiaceae, are sufficiently distinct to justify the establishment of a separate family in the same order.

#### TAXONOMIC

##### COELOMOMYCETACEAE, n. fam.

Parasitic in insects. Mycelium coenocytic, aseptate, without rhizoids or cell walls. Reproduction by thick-walled resting sporangia which develop from hyphal bodies in the insect's hemocoele. Resting body at first covered by a thin membrane formed from the old plasma membrane; wall proper consisting of two layers, the outer smooth or usually sculptured and brownish in color. In germinating, the outer membrane cracks and the spores are discharged surrounded by a gelatinous matrix. Zoospores posteriorly uniflagellate, with a rudimentary nuclear cap. Sporangial walls without cellulose.

##### *Coelomomyces* Keilin, emend.

Obligate parasites in insect larvae or adults. Mycelium rather poorly developed, without rhizoids or cell walls, surrounded only by a plasma membrane and hence absorbing its food directly over its entire surface; hyphae irregularly or rarely dichotomously branched, single threads of more or less uniform diameter, except tapering to a point at the posterior end or with irregular swellings and narrow places; contents consisting of densely arranged multinucleated cytoplasm, with small and inconspicuous vacuoles and numerous lipid granules. Giving rise to irregularly shaped hyphal segments by the enlargement of the end or a lateral branch of the hypha and its severance from the parent hypha by division or more frequently by the thinning down of the basal connection until

it is broken away by the movements of the insect's body. Thin-walled zoosporangia apparently lacking, but thick, pale yellow- to brown-walled resting bodies formed and behaving after a rest period as zoosporangia; sporangia formed as are the hyphal segments, and breaking away from the mycelium before the formation of a definite wall to complete development within the hemocoele of the larva or adult, finally completely filling the body cavity of the larva or adult, usually set free by the disintegration of the larva's or insect's body; surrounded by an exceedingly thin, hyaline, smooth, outer membrane derived from the old plasma membrane; wall two-layered, the outer layer usually the thicker, and smooth or pitted, banded, striated, ridged or otherwise ornamented. Dehiscing by a preformed longitudinal slit. Zoospore discharge and method of swimming much as in the Blastocladiales, structure as in that order except that the zoospores lack a typical and perfectly formed nuclear cap. Sexual phases apparently lacking.

#### **Coelomomyces dodgei, n. sp.**

Hyphae 7-14 $\mu$  thick, irregularly branched and anastomosing; sporangia 27-42 x 37-65 $\mu$ , oval, sometimes slightly flattened on one side, wall deep brown, rarely hyaline, 1.5-4 $\mu$  thick, consisting of an outer thickened hyaline to brown part (2-3 $\mu$  thick) and an inner thin hyaline layer (1-2 $\mu$  thick); outer part of wall with rounded or elongated pits or more frequently with long grooves, usually about 4 $\mu$  apart and about 1 $\mu$  wide, the grooves extending lengthwise or arranged in variously curved patterns, with 7-10 visible on surface view. The thickened part of the wall is thus laid down in parallel bands between the grooves. Wall with a preformed longitudinal groove along which dehiscence occurs to permit spore discharge. Germinating in water and without previous drying after the death and partial disintegration of the larva, the process occupying 24 to 36 hours. When ready to germinate, the contents swell, producing a lateral bulge and thus causing the thick outer wall to crack open at the longitudinal groove. Twenty-four to thirty-six hours later the spores emerge through this crack, surrounded by a gelatinous material which after three to five minutes disappears, allowing the spores to escape. Spores posteriorly uniflagellate, elongated and broadest at the posterior end, 2.6-3.8 x 5.2-6.3 $\mu$  when swimming, 4-5 $\mu$  when quiescent and rounded, with several lateral lypid granules and an indistinct nuclear cap.

Parasitic in larvae of *Anopheles crucians* (type of *C. dodgei*), *A. quadrimaculatus* and *A. punctipennis*; Dublin, Valdosta, and other locations in Georgia. Of the parasitic fungi so far found on mosquito larvae in Georgia, this is by far the most common. This species may be related to *Coelomomyces stegomyiae* Keilin, the type species of the genus, but the two may be separated by the size of the resting sporangia and the markings on the wall.

#### **Coelomomyces psorophorae, n. sp.**

Parasitic in the larvae of *Psorophora ciliata*; hyphae 7.5-10 $\mu$  thick, not well developed but breaking up into hyphal bodies which spread the infection through the host or develop into resting sporangia. Resting sporangia oval, frequently slightly flattened on one side, rarely spherical, 37-67 x 46-100 $\mu$  when oval; 40-78 $\mu$  thick when spherical; wall 3-10 $\mu$  thick, consisting of two distinct layers, the inner smooth, the outer with vast numbers of minute anastomosing pits and a longitudinal slit, both wall layers deep brown. Germination not observed.

This species has been found only once and then on one specimen of *Psorophora ciliata*, collected near Moultrie, Georgia (R 22), October 9, 1942. It has much the largest resting sporangia of any species of *Coelomomyces* yet described. It is a singular fact that the resting bodies in this species have a much thicker wall, up to  $10\mu$  thick, than any of the other species of *Coelomomyces*. This thick wall is undoubtedly an adaptation to the host's environment. Dyar (1928, p. 107) states that the genus *Psorophora* is dominant and conspicuous in semi-arid regions. The species are adapted to breed in the most transient of rain-water; the eggs have a spinose protecting coat, and may lie on the dry ground for months or years, hatching on the advent of water.

***Coelomomyces quadrangulata*, n. sp.**

Hyphae  $9.6-25\mu$  thick; sporangia roughly oval in outline but with numerous angularities, usually somewhat flattened on one side,  $12-21 \times 19-40\mu$ , in end or cross section view squarish or with four lobes; entire wall  $3-5\mu$  thick, consisting of two layers: the outer layer pale brown with four longitudinal ridges which give the spore the four-angled or lobed appearance in end or cross section view, with very minute, closely set pits which are frequently arranged in rows, and thus give the impression of transverse striations as in a diatom shell; the inner layer hyaline, circular; dehiscence by a preformed longitudinal furrow which runs along one of the ridges. Germination not observed.

This species is rather rare, occurring in four larvae of *Anopheles* sp. out of many examined. Collected in Georgia, November, 1944.

***Coelomomyces pentangulata*, n. sp.**

Hyphae  $4.2-10\mu$  thick, hyphal bodies very abundant and mostly round when first formed,  $12-15\mu$  thick; sporangia oval, elongated or wheat-seed shaped;  $12-18 \times 18-40\mu$ , most about  $14-31\mu$ ; wall  $2-3\mu$  thick. The outline of the sporangia in longitudinal section is usually smooth, though at times it may be slightly angular, the inner wall smooth or frequently undulating, with one to three or several wave crests on each side in section view. The two wall layers frequently separating at the two ends, or sometimes on the side, leaving clear spaces; surface with five to six elevated longitudinal ridges distinctly visible in cross section view as five or six elevations. Wall apparently very finely pitted, and with a distinct longitudinal slit on one of the ridges. Germination not observed.

This species is very common on *Culex erraticus*. Collected from Valdosta, Georgia, October, 1944. It is characterized by the small sporangia which in cross section view have usually five or six angles or elevations and hence the name *C. pentangulata*.

***Coelomomyces uranotaeniae*, n. sp.**

Hyphae  $4-12\mu$  thick, elongated, branched and anastomosing, rather sparse and poorly developed, frequently encased in a thick transparent sheath; hyphal bodies spherical, oval or pyriform; enlarging to an irregularly oval body,  $27-35 \times 30-55\mu$ , the protoplasm becoming differentiated into an inner, dense, oval, granular zone and an outer, wide hyaline zone; inner granular part  $12-31 \times 29-35\mu$ , outer zone  $4-12\mu$  wide; the resting sporangia developing from these bodies; resting sporangia oval in outline,  $21-30 \times 29-45\mu$ , with a very distinctive wall. The inner wall is smooth, hyaline and  $1.5-2.5\mu$  thick; the outer wall pale brown

or yellow and  $2\text{--}3\mu$  thick, extending out to form seven or eight longitudinal, anastomosing, steep ridges, about  $4\text{--}5\mu$  tall and spaced  $7\text{--}9\mu$  apart; wall between ridges with very fine transverse striations and entire sporangium covered with remains of the hyaline envelope which surrounded the structure when immature. In cross section view the ridges appear as seven or eight spines.

Found only once on *Uranotaenia sapphirina*, Thomasville, Georgia (B-4), October 9, 1944. This species is easily distinguished by the structure of its wall.

#### SYSTEMATIC POSITION OF COELOMOMYCES

As pointed out by Keilin (1921), the genus *Coelomomyces* belongs in the Phycomycetes since the thallus is multinucleate, lacks cross walls, and hence is coenocytic. The discovery of zoospores by McIlion and Muspratt (1943) would seem to remove any possibility of doubt that it belongs to the Phycomycetes. The writer, however, cannot accept the viewpoint of Keilin (1921, 1927) and Iyengar (1935) that the genus is related to the Chytridiales, but thinks that it shows a much closer relationship to the Blastocladiales. The reasons for this belief are as follows.

None of the Chytridiales have so thick and well developed a mycelium as is found in *Coelomomyces*. Where the mycelium is extensive as in the Cladophytaceae, it is very narrow. In the Blastocladiales, as in *Blastocladia parva*, some of Whiffen's (1943) figures (figure 25) strikingly resemble some of Iyengar's. Iyengar (1935) shows a dichotomously branched mycelium in both of his species, but does not mention this very important characteristic in the text. In several of the species described here, the mycelium shows a strong tendency to branch dichotomously as in *Coelomomyces quadrangulata*. The dichotomous branching is common in all the species of *Allomyces* and occasionally occurs in *Blastocladia*.

The wall structure of the resting sporangia in the Blastocladiales, particularly in certain species of *Blastocladia* and in all species of *Allomyces*, is like that reported for most species of *Coelomomyces*. In both groups the wall consists of two layers, an outer thicker, yellowish to brown, pitted or otherwise sculptured layer, and an inner, thinner, colorless, smooth layer. In the sporangia of *Coelomomyces*, Keilin (1921) indicated that in the outer sporangial wall there was a preformed line or fissure along which he suspected the sporangium would open during spore discharge. McIlion and Muspratt (1943) also observed this line and found that Keilin's guess that the sporangial wall would crack along this line during spore discharge was correct. This preformed line is a very characteristic and distinctive structure. I have observed it in four of the five species described here. In *Blastocladia simplex*, *B. asperosperma*, *B. laevisperma*, and *B. cystogena* several preformed lines along which the outer wall cracks may be seen. In *Allomyces javanicus*, *A. anomalus*, *A. arbusculus*, and *A. moniliformis* the outer wall of the resting bodies cracks in germinating along one or two longitudinal preformed lines, a fact of considerable importance in this genus which has not been previously noted. These lines of dehiscence are strikingly similar in appearance and function to the line in the outer wall of the resting sporangium of most species of *Coelomomyces*.

The details of spore development have not been completely worked out in *Coelomomyces* but the stages so far observed agree with the Blastocladiales. In *Allomyces* during sporogenesis and gametogenesis, Hatch (1935, 1944) and McCranie in Sparrow (1943) show the nuclei in a peripheral position at a certain stage of development. It is significant that Bogoyavlensky (1922) in *Coelomomyces notonectae* and Iyengar (1935) in *C. indiana* show stages in sporangial development in which the nuclei exhibit a similar peripheral arrangement.

Finally in *Coelomomyces* the method of spore discharge, the structure of the zoospore and method of swimming are in essential agreement with the Blastocladiales.

Another and perhaps very significant matter has to do with the comparative physiology of the resting bodies. In most species of the Blastocladiales, perhaps all, the resting bodies are incapable of germinating before undergoing a period of drying, and retain their vitality for a long time, up to several years in the dry condition. In *Coelomomyces* we can expect considerable variation in the resting sporangia in their relation to drying. In the species which parasitize mosquitoes, such as *Psorophora ciliata*, which breed in temporary pools in very dry habitats and whose eggs are capable of resisting drying for a long time, we may expect to find resting bodies capable of resisting prolonged drying, and from the extreme thickness of the wall of the resting sporangium of *Coelomomyces psorophorae*, it would appear that it was so adapted. On the other hand, the species of *Coelomomyces* on mosquitoes whose larvae inhabit permanent or semi-permanent water may have resting sporangia that do not require drying before germinating, and which indeed are killed by desiccation. Such appears to be the case with the resting bodies of *Coelomomyces dodgei* on *Anopheles crucians*.

While there can be little doubt that *Coelomomyces* is related to the Blastocladiales, its parasitism has been accompanied by such changes in its structure and life history that its position here is somewhat doubtful. It differs from the Blastocladiales and all other true fungi in lacking a cell wall while in the vegetative condition. It also appears to lack rhizoids, but absorbs its food directly through the plasma membranes. Bogoyavlensky (1922) was the first to notice the absence of a cell wall, and he considers the mycelium as plasmodial in nature and calls it a plasmodium, assigning the organism a place near *Sporomyxa scauri* Léger in the Plasmodiophorales. Keilin (1921, 1927), in describing *Coelomomyces stegomyiae* and in commenting on Bogoyavlensky's species of *Zogafia*, does not mention a cell wall on the mycelial threads and shows no signs of walls in the drawings of mycelia, hyphal bodies or young sporangia. Iyengar (1935) describes the wall of the mycelium as very thin and membranous.

The present writer is able to confirm the observations of Bogoyavlensky (1922) that the mycelium lacks cell walls. The evidence for the absence of walls is based on the following observations and simple experiments. No cell wall can be seen under the highest efficient magnification (20x ocular and 70x water immersion objective). This applies both to fresh material quickly dissected and immediately examined after mounting in water or 0.6% salt solution, and to material killed in 10% formalin and mounted in lactophenol and cotton blue.

For comparison, some actively growing threads of *Allomyces javanicus* were killed in 10% formalin, washed and mounted in the above lactophenol solution and thus treated exactly as the various species of *Coelomomyces* were treated in mounting. The wall in the *Allomyces* material was unmistakably clear, with the plasma membrane shrunken from the wall in many places. No such plasmolysis has been observed by anyone in the threads of *Coelomomyces*, though the sporangia after the wall has been laid down do show plasmolysis when preserved in 10% formalin. I have dissected larvae in water in whose living bodies the hyphae were very distinct, but only a few minutes after the hyphae come in contact with water they dissolve, leaving no trace of a wall. If such larvae are dissected in physiological salt solution (0.6% NaCl in water), the hyphal segments swell and disappear, but not so rapidly as in water. The cell wall substance of the Blasto-cladiales, so far as known, is not soluble in water. Indeed such a delicate endoparasitic species as *Catenaria allomycis* may be dissected out from its host in water, but its wall remains distinct. No fungus is known with hyphal walls that dissolve in water.

It is a fact worth mentioning that the hyphae, though lacking cell walls, retain a definite polarity and branch dichotomously or subdichotomously. This absence of cell walls on the fungal hyphae of *Coelomomyces* is a unique condition in the fungi. Except for the reported absence of a wall on the hyphal bodies of *Entomophthora colorata* Sorokine (cited by Thaxter, 1888), I know of no true fungal hyphae or hyphal segments that lack walls. It may be assumed that the hyphal bodies in *Coelomomyces* are capable of some amoeboid movement, as they are reported to have in *E. colorata*. However, I have been able to observe amoeboid or plasmodial motion of hyphal bodies only when in the fluid which exuded from a crushed insect mounted in water.

The life history of *Coelomomyces* is still incompletely known. A number of questions need to be answered: (1) whether the zoospores from the resting sporangia are the agents of infection; (2) how infection takes place; (3) whether infection is congenital; (4) what parts of the insect's body are attacked; (5) whether one species of fungus will parasitize more than one kind of insect; (6) whether there is an alternation of hosts; (7) whether there is an alternation of generations, i.e., an alternation of sporophytic and gametophytic plants as in certain species of *Allomyces*; (8) whether any of these fungi can be cultivated on artificial media; (9) whether any of these parasitic species can be used for artificial biological control of the insects.

#### SUMMARY

An historical account with a resumé of the species so far described is given for the genus *Coelomomyces* Keilin. Five new species, *Coelomomyces dodgei* on *Anopheles crucians* and other species of *Anopheles*; *C. psorophorae* on *Psorophora ciliata*; *C. quadrangulata* on *Anopheles* sp.; *C. pentangulata* on *Culex erraticus*; and *C. uranotaeniae* on *Uranotaenia sapphirina* are described from Georgia, the first report of this fungal genus from this hemisphere. From a study of living material of *Coelomomyces dodgei* and preserved material of this and the other

## EXPLANATION OF FIGURES

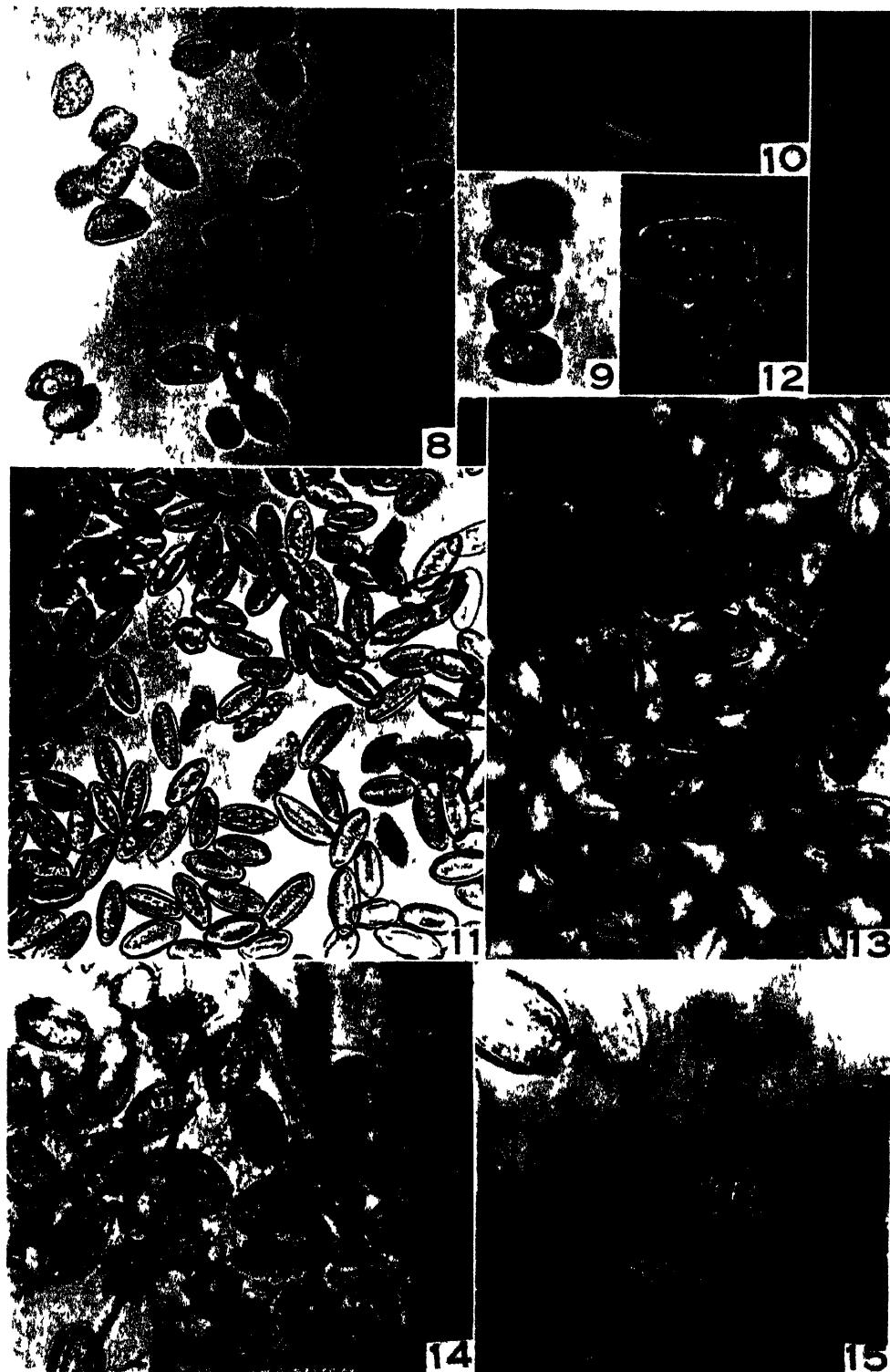
1. Head and thorax of larva of *Anopheles quadrimaculatus* containing oval resting sporangia of *Coelomomyces dodgei*.  $\times 32$ .
- 2 Resting bodies of various ages and some hyphal masses of *C. dodgei* from crushed insect of *A. crucians*.  $\times 400$ .
3. Branching hyphae and two resting sporangia of *Coelomomyces dodgei*, the immature one at top about ready to be severed from parent hyphae, on *A. crucians*,  $\times 300$
4. Empty resting sporangia of *Coelomomyces dodgei* showing wall structure, on *A. crucians*,  $\times 700$ .
5. Resting sporangia of *Coelomomyces psorophorae* on *Psorophora ciliata*,  $\times 200$ .
6. Resting sporangia upper surface, lower section view of pits in wall, *C. psorophorae*.  $\times 700$ .
- 7 Resting sporangia of *Coelomomyces pentangulata* on *Culex erraticus*, showing double layered wall,  $\times 1050$ .
8. Resting sporangia of various ages of *Coelomomyces quadrangulata* on *Anopheles* sp.,  $\times 400$ .
9. Four resting sporangia of *C. quadrangulata*, the two lower in end view showing four-angled shape,  $\times 600$
10. Mature resting sporangia of *C. quadrangulata* in longitudinal section view, showing thick double wall,  $\times 1050$ .
- 11 Resting sporangia of various ages of *Coelomomyces pentangulata* on *Culex erraticus*  
Note ones with no cell wall,  $\times 400$ .
- 12 Two resting sporangia of *C. pentangulata* on *C. erraticus*, one side view, other end view, latter showing ridges,  $\times 1050$ .
- 13 and 14. Resting sporangia of *Coelomomyces uranotaeniae* on *Uranotaenia sapphirina*  
Note ridges which in section view appear as spines In fig 14, top center, note immature resting sporangium surrounded by halo, and in lower right a few fragments of hyphae,  $\times 400$
- 15 Resting sporangia of *C. uranotaeniae* showing ridges and very indistinct transverse striations between ridges,  $\times 700$

PLATE 1





PLATE 2





# STUDIES ON THE ABSORPTION AND METABOLISM OF QUININE AND RELATED PROBLEMS

BY JAMES C. ANDREWS AND GRANVIL C. KYKER

*Department of Biological Chemistry and Nutrition, School of Medicine, University of North Carolina*

During the past six years this department has been engaged in an active program of research with the object of attaining a more exact knowledge of the physiological mechanism by which quinine acts on the malaria parasite. Such studies were the result of a desire to improve the efficiency of the application of quinine and to obtain information which might lead to possible synthetic substitutes, more satisfactory than those now available. At the time of their inception in 1939, these studies were not actuated by the urgencies of the present war situation and the sharp shortage of quinine during the past few years has somewhat shifted the emphasis of our work. One of our early points of interest resulted from an impression on the part of certain physicians and public health officers in the more malarious districts that the simultaneous presence of malaria and of hookworm infection in the same patient caused a resistance on his part to quinine therapy as ordinarily applied. Such a condition is naturally hard to demonstrate in any quantitative way but we felt it worth while to seek for any possible influence which the hookworm with its resulting damage to the small intestine, might have on the levels of quinine in the blood resulting from the absorption and subsequent metabolism of therapeutic doses of the drug.

The program necessarily depended for its validity upon a method for the reliable determination of the small amounts of quinine which are present in the blood stream after ingestion of therapeutic doses. These amounts range in value from zero up to a maximum of 10 to 12 milligrams per liter of blood or parts per million. The determination of such small quantities obviously lies in the range of micro-chemistry. We first made a careful scrutiny of those methods already on record in the literature. These, if applicable to such micro technique, must be based on the production of a colored compound of high tinctorial power, the formation of a highly insoluble precipitate, or the production of some highly delicate phenomenon such as fluorescence. In all such cases standard solutions of quinine of known purity are essential for comparison and one of the first problems involved is to obtain or to prepare pure samples of quinine free from contamination with allied cinchona alkaloids. Most commercial samples of any of the common cinchona alkaloids are highly contaminated with related members of this group. The most reliable criterion of complete purity is the attainment, on repeated recrystallization, of constant solubility and constant optical rotation. The latter is of particular value but must be carried out under sharply defined conditions since the optical activity of even a pure sample of a cinchona alkaloid varies with the solvent, the temperature, the nature of the salt employed, etc. Some preliminary studies were therefore necessary on the optical

activity of quinine and other cinchona alkaloids and these were made at an early stage of the work (9, 3). Of particular interest in this connection was the question of the effect of change in solvent of water to ethyl alcohol in varying percentages on the resulting optical activity of the solution. It was found that in practically all cases certain mixtures of alcohol and water gave optical activities, for the same amount of the cinchona alkaloid, much higher than when either pure water or pure alcohol was used. Much of the older literature on this subject has been published without specification of these conditions but when such determinations are to be used as a criterion of purity, the need for such specification is obvious.

As stated above, the successful pursuit of a biological study of the mechanism of the action of quinine ultimately depended upon a quantitative analytical method for small amounts of the alkaloid. Numerous methods have been proposed at one time or another. These methods are based on one of the following general types of analysis: titration (19), colorimetry (40), turbidimetry (38), nephelometry (42) and fluorimetry (20). Most individual procedures require an extraction of the alkaloid from its source or from the biological sample containing it before its determination can be accomplished. No method had been proposed before the beginning of this work which offered the sensitivity, reproducibility and rapidity which were anticipated to be essential to our proposed plan but the method which had been proposed by Vedder and Masen (42) gave evidence of being as desirable as any other which was available at the time. A critical study of this method by Kyker, Webb and Andrews (34) resulted in its modification and adaptation to modern instrumentation. The procedure which evolved from this work consists in the continuous extraction with ether of the quinine from a sample which is adjusted to proper alkalinity, the taking up of the extracted quinine in dilute hydrochloric acid (0.030N), the preparation of a turbidity by treating the dilute acid solution of extracted quinine with silicotungstic acid, and the determination of the density of the silicotungstate turbidity by means of an Evelyn photoelectric colorimeter. The method requires the establishing of a reference curve which results from plotting the instrument readings as a function of the concentration of a series of standard quinine solutions. Triplicate samples were used in establishing the accuracy of the procedure. A 5 ml. sample is sufficient for the triplicate determination of quinine in blood or urine by the micro procedure within  $\pm 4\%$  error at levels as low as 1 mg. per liter. The satisfactory development of this method required detailed studies of the following factors: (a) the solvent used in the extraction of the alkaloid; (b) a new apparatus and method for continuous extraction; (c) the effect of the concentration of the hydrochloric acid in which the turbidity is prepared, on its stability; (d) the filtration of quinine through various materials and the accompanying loss by adsorption; (e) the effect of various physical factors in the preparation of quinine silicotungstate turbidities; (f) the spectral transmission of quinine, silicotungstic acid, and quinine silicotungstate at various wavelengths from 350  $\mu$  to 950  $\mu$ . By the use of a unit of six extractors, it has been practicable for one operator to analyze in one day as many as twelve biological samples which contain quinine.

Each of the numerous methods which has been proposed for the estimation of quinine has the disadvantage of non-specificity, although many of these methods may be sufficiently accurate for rigidly controlled biological work. It is possible for a normal biological sample of tissue or excreta to give a blank result for quinine by a certain analytical procedure after which one may find that the results by the same method on a similar sample, which has been selected after the administration of a dose of the alkaloid, are vitiated by the presence of one or more interfering substances which have resulted from the partial degradation of alkaloid by the body.

In an effort to clarify the interpretation of measurements of quinine by the method which we have proposed (34), to define related substances which interfere (29), and to extend the application of the procedure to the assay of certain other substances, Kyker and Lewis (32) have studied a wide variety of compounds which have some properties more or less in common with quinine. Those compounds which were studied are classified as follows: (a) cinchona alkaloids (cinchonine, cinchonidine, hydroquinidine, quinine, quinidine, quitenine, and totaquine); (b) other alkaloids (caffeine, cocaine, colchicine, morphine, narcotine, nicotine, strychnine, and theobromine); (c) synthetic antimalarials (atabrine and plasmochin); (d) compounds similar in structure to part of the cinchona nucleus (pyridine and quinoline); (e) vitamins (choline, nicotinic acid, pyridoxine, riboflavin, and thiamine); and (f) miscellaneous nitrogenous substances (antipyrine, cinchophen, novocaine, sodium barbital, proteose-peptone, egg albumin, and serum albumin). Multiple samples of certain of the cinchonas, which were obtained through different sources, were studied since most commercial samples of cinchona alkaloids may be adulterated more or less with another and since it was anticipated that one cinchona might give results similar to another. A sample from one or more sources of each of the above compounds was subjected to the procedure for quinine determination and the results compared with those for quinine. Also, the composition of the silicotungstate turbidity was determined for twenty of the bases, and the optical rotation of the optically active substances was studied under conditions not previously recorded. Nicotine, thiamin, and all of the cinchonas except quinidine give silicotungstate turbidities which are similar in properties and density to that produced by approximately the same concentration of quinine. Also at slightly higher concentrations, the turbidities which are derived from novacaine, strychnine, narcotine, cocaine, quinoline, serum albumin, egg albumin, and proteose-peptone are similar to those just mentioned. Only at concentrations which are relatively very high do antipyrine, morphine, caffeine, pyridine, and choline yield insoluble silicotungstates. The remaining substances are either not adaptable to this study or else give no response to this treatment. Quitenine, thiamine, cocaine, and the proteins, which respond to the reagent in a manner similar to quinine, offer no interference in the use of the method for quinine because these are not extractable during the continuous ether extraction.

The intensification of effort which the war has laid on the study of the cinchonas has placed emphasis on the finding of accurate methods which are even more rapid and simple to perform. As a result a number of methods (26, 11, 13)

have appeared which employ different solvents and techniques. Prior to the appearance of these we were in the process of improvement of our method. The extraction apparatus was modified (30) to accomodate the use of a heavier than water solvent. The use of this apparatus with chloroform permits the complete extraction of quinine from a biological sample in 15-30 minutes (36) instead of the 4 to 5 hours prescribed for the continuous ether extraction. The extracted alkaloid may subsequently be estimated either by the silicotungstate method or by fluorimetry for which the Coleman photofluorometer was used. An inspection of the data which have been reported from various laboratories, on the distribution of a dose of quinine throughout the animal body shows a conspicuous lack of information on the content of quinine in the lower intestinal region. Our studies by the ether method of extraction show the presence of significant amounts of some unknown interfering substance from such samples whereas the chloroform extraction shows more promise of specificity in this respect.

With the method for the determination of small amounts of quinine in blood in satisfactory condition, it was applied to the measurement of levels of quinine attained in blood and urine of dogs following single doses of quinine sulfate, both when the dogs were normal and free from intestinal parasites and when the same animals had been given a moderate hookworm infection (10). These experiments were then repeated after the same animals had been freed from the parasites by treatment with anthelmintics. Blood and urine collections were made at frequent intervals and curves were published showing the changes in the concentration of quinine in both up to 24 hours from administration of the dose. The results may be summarized as follows: Hookworm infection, with the resulting damage to the small intestine, did not decrease the maximum quinine level attainable in the blood nor did it materially change the time required to attain that maximum. There were slight indications that in some cases the shapes of the curves of quinine concentration in the blood versus time differed somewhat from the normal in the occurrence of a plateau following the original peak. Since the drop in blood concentration following the peak is to be ascribed to the combined effects of urinary excretion of quinine and metabolic decomposition of the drug and since the rate of urinary excretion should be roughly constant, it seemed reasonable to conclude that when these plateaus occurred, they were the result of impaired metabolic decomposition of the drug. It was considered possible that such impairment might have been the result of the anemia produced by hookworm infection and an investigation of this point was planned. However, it was evident from these studies that no impairment of the absorptive mechanism could be demonstrated under the conditions of these experiments and that, as a result, the original hypothesis of defective absorption by a damaged intestine could be discarded.

To determine if any relationship existed between hemorrhagic anemia and the levels of quinine attained in blood and urine, two dogs, freed from intestinal parasites, were used as described above (5). After a preliminary absorption experiment under normal conditions, hemorrhagic anemia was produced by heavy bleeding. After hematocrit values of not over 20% had been attained

for two weeks, the quinine administration was repeated. The shape of the resulting curves varied from the normal in that a somewhat higher level of quinine followed the initial peak. These curves were sufficiently similar to those reported after hookworm damage to indicate that the results of Andrews and Webb were probably the result of the anemia that accompanies hookworm damage. No significant change was found in the percentage recovery of quinine in the urine.

In the studies of Andrews and Webb the percentage recovery of quinine in the urine of the first 24 hours varied from 4 to 12% of the dose administered. These low figures might well have resulted from a high degree of metabolic decomposition but the suspicion persisted that the rate of absorption of quinine sulfate, although apparently not affected by hookworm damage, might in all cases have been so low as to permit considerable fecal loss of the drug. For this reason a different type of experimentation, in which fecal loss was prevented, was devised to answer this question. Dogs with isolated intestinal loops were prepared by the method described by Johnston (25). These loops, entirely separated from the remainder of the intestinal tract, have their blood and nerve supply intact and can be used for repeated measurements of absorption. One end of the loop is closed while the other is brought to the abdominal wall. Various substances can be introduced into it, left for a given period of time and the residue then washed out in fairly quantitative fashion. Thus there is attained a measure of the amount of the drug actually absorbed into the blood stream and all possibility of fecal loss is prevented. Although such loops represent only a fraction of the total absorbing surface of the animal they make possible more accurate comparisons between different compounds since repeated determinations can be made on the same animal. Since the isolated loops vary in size and position from one animal to another the results in terms of absolute percentage absorption can vary widely. However, for any one animal they can give very accurate comparisons of one compound with another or of one compound under varying conditions.

Some preliminary results of this type were published by Andrews and Anderson (4) and a more detailed study by Cornatzer and Andrews (15). Some of the results obtained may be summarized as follows: The more soluble quinine dihydrochloride is absorbed more rapidly than the sulfate while the free base was absorbed more slowly than either. The use of sodium chloride solution with quinine sulfate, while more than doubling the solubility of the latter, caused no increase in the rate of absorption. In no case did the amount of the drug absorbed bear a linear relation to time, since the rate of absorption decreased greatly during the later periods of a prolonged experiment. Attempts to quantify the amount of quinine absorbed from the intestinal loop in terms of that present in the blood and excreted in the urine showed that at least 75% of the drug remained unaccounted for, even in experiments with urine collections lasting 48 hours. However, this series of measurements showed that absorption of the drug cannot be the limiting factor, since even with the small proportion of the animal's total absorbing surface used, blood levels of quinine were obtained comparable to those found by Andrews and Webb (10) for the whole intact

animal. *Cornatzer and Andrews* also determined the relative rates of absorption of some of the other common cinchona alkaloids such as cinchonine, cinchonidine, etc. These also have some antimalarial value and are absorbed at rates comparable to those of quinine.

A further series of studies was made of the effect of administration of alkalies and acids on the absorption and metabolism of quinine. At various times in the past the simultaneous administration of alkalies with quinine sulfate or dihydrochloride has been recommended on the ground of increased absorption and improved clinical results (1). On the other hand verbal claims have been made that refractory cases of malaria sometimes yield to quinine sulfate therapy if therapeutic doses of hydrochloric acid are administered with the quinine. In neither case have the authors been able to find acceptable proof of these statements. Our experiments with "loop dogs" have shown that if enough alkali is administered with the quinine salt to convert it into the free base a lowered rate of absorption, corresponding to the latter is produced. However, in most cases the added alkali, converted to bicarbonate in the small intestine, is so rapidly absorbed as to permit very little reaction or contact with the quinine salt. On the other hand, ordinary therapeutic doses of hydrochloric acid have very little effect on the pH of either a normal gastric juice or of the small intestine, and increases in acidity within the limits possible in the intestinal loops have produced no increase in rate of absorption in our experiments.

A Russian investigator (23) has reported a lowered urinary excretion of quinine resulting from alkali administration and has therefore recommended alkali therapy on the somewhat dubious ground that what is not excreted is retained and is therefore capable of producing more antimalarial effect. Such a suggestion, of course, completely neglects the question of changes in metabolic decomposition of the drug. Haag, Larson and Schwartz (24) have confirmed quantitatively this finding that with equal doses of quinine, acid therapy causes more to be excreted and alkali therapy less. The same finding has been confirmed by Andrews and Cornatzer (7) using the dogs with isolated intestinal loops. In these latter experiments acid administration to the animals caused urinary elimination of about double the amount of quinine as did alkali administration.

Since the search for the mutual relation of hookworm and malaria did not provide the solution to the problem it was postulated that malnutrition, which in general could be assumed to be coincident with the economic and sanitary status prevailing in the average endemic hookworm area, may affect either the vitality of the parasite or the mechanism by which the antimalarial drugs act. Since certain members of the B-complex are known to conjugate to produce specific metabolic catalysts and since these vitamins, like quinine, possess constituent nitrogen, they were thought to be the most likely of the vitamins to suspect in any vitamin-drug inter-relationship. Little has been written concerning the mutual effect of vitamins on drugs and it is conceivable that either the vitamins may be utilized in the degradation of quinine which is largely destroyed by the body or that quinine with its nitrogen structure similar to the

nitrogen of certain B vitamins could be confused with the vitamins by the metabolic processes of the body in a manner somewhat analogous to the p-aminobenzoic acid and sulfonamide relationships which are thought to prevail in the living processes of certain micro organisms. The portion of a dose of quinine which escapes degradation and is excreted unchanged may be different when comparisons are made between adequately fed and vitamin deficient animals if the postulation is justified and if the magnitude of the effect is detectable by the methods which are applied. All of the studies which concern vitamin relationships were done on rats. When tested experimentally (28) it was found that the progressively increasing B-complex deficiency symptoms were accompanied by a progressive decrease in the percentage of the oral dose of quinine which was excreted by the kidney. The minimum level of excretion obtained for almost a week after the inadequate diet was supplemented with liver and yeast and then the excretion rose rapidly. Following this, the excretion levels fluctuated irregularly from high to low values. The regular decrease in excretion in the earlier phases of this experiment was not observed when in a similar group of subjects the combined urinary and alimentary excreta were collected and analyzed. If the absorption of quinine is influenced by the deficiency of vitamins the results can be correlated. Consequently, experiments were devised which would reflect separately the effects of the vitamin deficiencies on absorption and the effects on metabolism. The former was measured by an adaptation of the Cori technique (14) and the latter was measured by the excretion of a parenterally administered dose of the drug which by-passed the site of absorption.

The adaptation (33) of the Cori method for the study of intestinal absorption consisted in an alkaline digestion and solution of the alimentary tract and the analysis of an aliquot of the digestate whereas Cori washed out the intestinal contents and analyzed. The adaptation was judged to allow for more rapid and accurate work since the quantitative washing out of unabsorbed quinine is technically difficult to duplicate. The amount of quinine in the tissue of the intestinal wall is very small and adds a negligible amount to that which remains in the contents of the intestine. The results of the study indicate that: (a) there is no difference in the absorption rate of quinine by normal and vitamin B-complex-deficient rats, (b) there is a decrease in the absorption of quinine as a function of time in both normal and deficient animals, and (c) there is at any given time after dosage a larger amount of the dose remaining in the stomach of the normal animals. The first conclusion is a contradiction of the result one would anticipate from similar studies on the absorption of sugars (21, 35). Also, in the case of many food materials, the rate of absorption maintains itself over a period of time rather than decreasing in rate as it was observed here. The last is opposite to the conclusion one would reach from studies on the effect of vitamins on gastric motility (22). These conclusions are substantiated by such strict uniformity in observations that their contradiction of previous reports demands an interpretation which superimposes the pharmacological influence of the drug itself on the factors which govern the processes of absorption.

The studies to determine the effect of avitaminosis-B on the metabolism of

quinine consisted in measuring the percentage of excretion of un-metabolized quinine which has been introduced parenterally by either subcutaneous or intra-peritoneal injection (36). In repeated series of animals there was no significant difference in the average excretion of quinine between the normal and deficient groups. The results of the observations on the separate processes of absorption and metabolism are difficult to correlate with the results of the experiments which combine both, through the oral administration of quinine. The question remains under further consideration.

All of the work done on quinine metabolism, both in this and other laboratories, has emphasized the fact that an overwhelming proportion of the drug which is absorbed into the blood stream is decomposed into products that are not definitely characterized. Moreover, the fact that malaria-infected red cells *in vitro* are very little affected by concentrations of quinine even if much higher than those normally attainable in the blood stream has led to constant suspicions that the real antimalarial action is a result of some metabolic product of the quinine rather than of the undecomposed drug itself. Waletzsky and Brown have reported experiments which contribute evidence to this mechanism on the indirect action of quinine (43). For this reason there have been various attempts to study the metabolic decomposition of this drug, both as regards the identity of its products and as regards the site of this decomposition. Concerning the latter point studies by Andrews, Cornatzer and Anderson (8) have demonstrated that the site of this decomposition is largely in the liver. This work was done partly with rats and partly with dogs. In either case standardized oral doses of quinine were given and quantitative urine collections were made. The same series of rats was then subjected to partial hepatectomy at which time from one-third to one-half of the liver was removed. The excretion of quinine was again measured with the result that this excretion increased from 72 to 143 per cent over that observed with the same preoperative animals. When the livers of these animals were allowed to regenerate after four to six weeks a third administration of quinine was found to be excreted to about the same degree as in the original normals. The effect of the removal of the liver in decreasing the extent of quinine decomposition was thus demonstrated.

A similar type of experimentation was carried out with dogs but in this case the livers were damaged by chloroform anesthesia. Normal animals were given standard dosages of quinine by mouth, after which blood levels and urinary excretions of the alkaloid were measured as described by Andrews and Webb (10). After two such experiments the animals were put under chloroform anesthesia for 30 minutes. Forty hours afterwards, quinine was again administered and blood and urine levels were followed as before. The same experiment was also carried out 112 hours after the time of anesthesia. Following this, the animal was subjected to chloroform anesthesia for one hour, and two quinine administration experiments 34 and 82 hours afterwards were run. Four months afterwards, when normal liver conditions had been reestablished, a last dose of quinine was administered, followed by the same determinations as before. During the periods of acute liver damage, definitely higher levels of quinine were maintained

in the blood stream and the percentage recovery of quinine in the urine was roughly doubled, the amount of increase being dependent on the length of the period of anesthesia (8).

Quitenine has been suggested as a possible end product of quinine metabolism. In this compound the chief structures remain intact but the vinyl group has been oxidized to a carboxyl group, thus producing an amphoteric compound with properties markedly different from that of the original quinine. Although quitenine has been demonstrated to have no antimalarial properties, certain of its alkyl esters have a moderate degree of such activity (12). Because of lack of much information concerning the properties of quitenine some studies of this compound were published by Andrews and Cornatzer, particularly as regards its dissociation constants and its optical activity (6). The data in this paper indicate however that quitenine can hardly play an important part as a metabolic oxidative product of quinine.

Some results from this and other laboratories have indicated that the metabolic decomposition of quinine is an oxidative procedure catalyzed by an enzyme which has been partially purified and, to a certain degree, characterized. This preparation, however, while moderately active, has exhibited certain properties which are very unusual. For example, the substance is so thermostable as to make it somewhat doubtful whether it can properly be called an enzyme at all. Thus far only results of a very preliminary nature have been obtained, and further investigation of this so-called enzyme and its properties is necessary (2).

Some preliminary studies of the properties of a metabolic oxidative product of quinine have been made by Andrews and Valk (41) and this work has succeeded in establishing to a certain degree curves for the ultraviolet absorption of this compound, together with certain other of its properties. While these studies were in progress publications by Kelsey, et al. (27) and by Mead and Koepfli (37) appeared describing the composition and properties of a metabolic product obtained from enzyme treatment of quinine. It is obvious that much investigative work remains to be done before we can speak with any certainty concerning these products of metabolic decomposition. It has been a hope of various investigators of quinine chemistry and metabolism that the identification and study of such metabolic products might lead to improvements in the efficiency of administration of the drug and might even point the way to more efficient synthetic substitutes than exist at the present.

The stability of quinine solutions and standards to exposure to light has been mentioned at different times in earlier work and we have reported brief experiments (34) which confirm these statements. Neither the course of the photochemical decomposition nor the extent to which it takes place under specified conditions has been studied previously. Experiments were carried out which were designed to show the stability of equimolar concentrations of quinine in different concentrations of hydrochloric acid when the solutions are exposed to sunlight (31). Since no analytical method for quinine is absolutely specific the progress of the photochemical degradation of the alkaloid was followed by our silicotungstate method, before and after extraction, and by the specific rotation.

The results by the different methods of analysis did not always agree quantitatively but did agree qualitatively. The results lead to the conclusion that the greatest stability in hydrochloric acid is provided at 0.03 M and that the decomposition which results from equal exposure to sunlight increases as the acid concentration decreases to zero and increases to 2 M.

Roskin and Romanowa (39) have reported an enhanced antimalarial effect of quinine by ultraviolet irradiation from an artificial source. Their comparison of the antimalarial activity of irradiated with non-irradiated quinine solutions was made with the canary. The biological testing of certain of the products which have been derived in this laboratory by exposure to sunlight has been done with *P. lophurae* in Pekin ducklings. Reports on this work (16) are in the process of preparation and for the present it is sufficient to say that the antimalarial activity of the irradiated solutions does not decrease proportionately with the concentration of quinine. Consequently one is led to the position that there is a photo decomposition product which retains its plasmocidal activity and loses its specificity to the method of analysis or which retains its response to the analysis and increases in antimalarial activity. Further work on this problem is in progress.

While investigating various problems connected with the administration, absorption and mode of action of quinine this laboratory has been interested in many other plant products of possible antimalarial value. The principal one of these is a Central American plant by the name of *Rauvolfia heterophylla*. This plant has already been described in the literature and has even been characterized as containing alkaloids of antimalarial value (18). However, tests made in this laboratory with adequate controls on colonies of ducks infected with bird malaria (*P. lophurae*), have shown completely negative results for this compound (17).

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# DARK-FIELD MICROSCOPY OF SURFACE PHENOMENA IN PHOSPHOLIPID FILMS AND IN FORMED ELEMENTS OF BLOOD AND BONE-MARROW

BY JOHN H. FERGUSON

*Department of Physiology, University of North Carolina*

## PLATES 3-5

Siedentopf and Zsigmondy (1), applying the optical principles worked out by Abbé and Helmholtz, first used the dark-field method of microscopy, which was quickly applied to the study of living cells and micro-organisms by numerous investigators in Germany, France and elsewhere. The early history of the use of this method for the study of the blood is cited by Aynaud and Jeantet (2). In a previous publication (3), dealing with the phenomena of platelet alterations, data were advanced in support of the thesis that the peculiar breakdown of these formed elements, *in vitro*, has an important relationship to the behavior of the lipids at the protoplasmic surface. The present communication represents an extension of these studies to other cell types and to certain artificial water-phospholipid films.

*Platelet alterations.* The chief findings with regard to the *in vitro* alterations of mammalian blood platelets (3) may be briefly reviewed. In citrated (0.38 per cent) or oxalated (0.1 per cent) blood plasma, the platelets are preserved but do show a gradual protrusion from the periphery of semi-rigid "excrescences," the formation of which is retarded by absence of contact with wettable surfaces, e.g., by keeping the plasma in paraffined tubes (4). In recalcified (N/10  $\text{CaCl}_2$ ) plasma, a peculiar lysis of the platelets quickly occurs, the chief characteristic of which is a vesiculation and disruption of the excrescences and the body of the platelet. The most typical appearances are seen in an untreated drop of blood allowed to coagulate on a microscope slide under the dark-field microscope. After a preliminary swelling, "spreading," and clumping of the platelets, their margins are seen to develop delicate excrescences, which may be described as filamentous, club-like, and vesicular (spheroidal). Not infrequently these become detached, but they usually persist in close relation to the granular residue (body) of the platelet. The "vesicular" forms are most numerous and they often contain refractile granules exhibiting a dancing type of Brownian movement.

Fig. 1 shows the semi-rigid excrescences of rabbit blood platelets preserved in citrate, the typical appearance usually referred to as "stellate" forms. Fig. 2 shows lysing forms of same, on the addition of calcium salt. Note the vesicular appearance of the platelets and the coincident appearance of fibrin threads in the coagulating plasma. Fig. 3 shows the platelets (and other formed elements) in the coagulated drop of human blood. Note again the "vesicular alterations" of the platelets. Fig. 4 shows that vesicular lysis of platelets (e.g., by saponin) may be induced without initiating plasma coagulation.

*Megakaryocytes.* Bone-marrow, freshly obtained from the femur or tibia of anesthetized rabbits, is quickly mounted in serum previously obtained from heart-puncture blood of the same animal. The dark-field microscope reveals "vesicular excrescences" (Fig. 5) developing at the periphery of a small proportion of the bone-marrow giant-cells, notably those of the *megakaryocytic* (not polykaryocytic) type. These vesicles, containing numerous granules oscillating with Brownian movement, are apparently identical with the similar alterations of blood platelets and, like the latter, they frequently become detached. The inability, in a large series of extended observations, to detect actual fragmentation of megakaryocyte protoplasm into whole platelets is noteworthy but not significant in view of the evidence for the correctness of Wright's data concerning this origin of the platelets, including one or two reports of the *in vitro* fragmentation for which we looked in vain (5). When bone-marrow is preserved and mounted in 3.8 per cent (isotonic) trisodium citrate, the megakaryocytes show a well-defined outline and fixed 'glassy' appearance of their very granular cytoplasm, together with suppression of the slow ameboid movement which can ordinarily be detected. These findings closely resemble the appearances of leucocytes in citrated blood. On recalcification, the megakaryocytes develop a few "vesicles" (Fig. 6) of somewhat smaller size but greater refractivity than those in Fig. 5, and they are almost devoid of granules. Saponin (in a limited series of observations) appears to increase the number of vesicles in recalcified megakaryocytes, but does not seem to be effective in the absence of calcium ions.

*Thrombocytes.* The nucleated thrombocytes of frogs, turtles, fresh-water sun-fish, and fowls have been studied under similar conditions. As frequently noted in the large literature on these formed elements, the normal *in vitro* disintegration of thrombocytes is often very sudden and "explosive" in character, scattering granular trails of material for some distance into the surrounding plasma from which fibrin is deposited, usually along these trails and frequently through the residual body of the corpuscle. It is highly significant, however, that "vesicular" bodies, essentially similar to those described for platelets and megakaryocytes, frequently persist around the thrombocyte remnant (Fig. 7). Furthermore, citration prevents the thrombocyte breakdown, and subsequent recalcification (Fig. 8) causes its reappearances with an even more complete and explosive violence than before.

*Stromatolysis of Erythrocytes.* Furchtgott (6) made dark-field observations of altering erythrocyte "ghosts" (hemolyzed red cells) in contact with lyotropic salt solutions such as lithium perchlorate or potassium thiocyanate. His "stromatolytic forms" (like our platelet alterations) were compared with the "myelin forms" of phospholipid films, to which reference will be made in a subsequent section. His search of the literature revealed several earlier observations of stromatolytic forms from both hemolyzed and non-hemolyzed red cells. In some unpublished observations extending back to 1930, we have also noted these forms and herewith reproduce three photomicrographic illustrations. Fig. 9 shows a few "filaments" extending from young erythroblasts in a drop of heart-blood from a rabbit embryo. Fig. 10, for which we are indebted to an erstwhile

colleague, Dr. P. H. Ralph (now at Ohio State University), is an excellent example in a nucleated red cell of the tadpole. Fig. 11 (courtesy P. H. Ralph) shows thread-like processes connecting points at which normal human red cells have apparently touched and then withdrawn from one another. This is evidence for a role of contact adhesion (fundamentally related to the surface-tension-lowering effects of "wetting") in causing filamentous developments from the cell membrane. The essential similarity of these phenomena to certain of the platelet alterations is noteworthy. Furchtgott (op. cit.) gives the additional information that "ghosts," made of hypotonic lysis and then extracted with chloroform, do not give the stromatolytic forms.

#### MYELIN FORMS AND RELATED PHENOMENA IN PHOSPHOLIPID FILMS

*Earlier data.* Virchow (7) first described "myelin forms (figures)" as microscopical appearances at the edge of nervous tissue surrounded by watery solutions. He interpreted the phenomena in terms of surface (interface) relations between the lipoid (fatty) and aqueous phases.

Leathes (8) photographed, at successive half-minute intervals, the large myelin forms appearing at the edge of a film of "lecithine" in contact with a wide variety of aqueous solutions, chosen to illustrate the effects of (a) specific ions, (b) osmotic concentration, (c) adsorbed substances. The myelin figures were readily produced by distilled water. *Inhibitory* influences included (1) Ca-ions, e.g., N/100  $\text{Ca}(\text{OH})_2$  and saturated  $\text{CaCl}_2$  solution: these caused complete suppression of the myelin forms, but this effect could be overcome by small amounts of N/100 NaOH and by cholesterol, in the case of the lime water, and by dilution (100x), in the case of  $\text{CaCl}_2$ ; (2) very strong (saturated)  $\text{MgCl}_2$ ,  $\text{NaCl}$ ,  $(\text{NH}_4)_2\text{SO}_4$  solutions: the inhibition generally being incomplete; (3) egg-albumin, gelatine, and saponin: also merely retarding. The chief *favoring* influence appeared to be dilution and good myelin figures were obtained with (1) N/100 acids, esp. HCl; (2) N/100 alkalies, esp.  $\text{NH}_4\text{OH}$ , also NaOH, KOH,  $\text{Ba}(\text{OH})_2$ ,  $\text{Sr}(\text{OH})_2$ ; (3) ordinary salt solutions, incl. Ringer's fluid (with or without  $\text{CaCl}_2$ ) and phosphate buffer mixtures (pH = 6.3-7.7); (4) human serum and hemoglobin (hemolyzed erythrocytes); (5) cholesterol-containing lecithin. Leathes correlated these data with the expansion of monomolecular films (cf. Langmuir, Harkins, Adam) and attempted an explanation in terms of the hydrophilic properties of the glyceride and amino portions of the oriented phospholipid molecules. He concludes: "myelin forms are due to surface growth and localized inhibition" [of water].

Ferguson (3) suggested a correlation between blood platelet alterations and myelin figure formation on the basis of some dark-field observations on egg-lecithin. The following facts were noted: "a, an *initial* myelin figure formation occurs immediately with all watery solutions [N.B. the strongest  $\text{CaCl}_2$  solution used was M/20] and is followed by a further expansion after a delay period; and b, the secondary phenomena are profoundly influenced by the demonstrable alternatives of i, outflow and solution of the lipoid into the watery phase, or ii, penetration of water into the lipoid phase, the latter occurring in the presence of calcium ions, which explains the "translucent border" noted by Leathes."

Furchtgott (6), likewise, correlated the stromatolytic forms of red blood cell "ghosts" with dark-field observations on the myelin forms from commercial lecithin (and sciatic nerve myelin) treated with NaCl or KCNS.

The present data extend similar observations to a study of *cephalin* and related brain phospholipids.

#### EXPERIMENTAL

*Reagents.* An opportunity to investigate the significance of type of phospholipid was afforded through the courtesy of Dr. J. Folch Pi (Rockefeller Institute, N. Y.) who supplied samples of four phosphatides, fractionated from brain "cephalin" and described elsewhere (9). These specimens, several years old, had the usual brownish color of phospholipids exposed to atmospheric oxygen. No exhaustive study of the myelin figure and related phenomena is here contemplated, the choice of salt solutions being determined solely by considerations with respect to (a) osmotic strength and (b) specific  $\text{Ca}^{++}$  and  $\text{Na}^+$  effects. N/7 solutions were selected as equivalent to a "physiological isotonicity" between that of cold-blooded animal Ringer's fluid and mammalian Locke's solution. A 142:5 mixture of the equinormal  $\text{NaCl}$  and  $\text{CaCl}_2$  solutions provides the two cations in the proportions in which they occur in normal human serum (Gamble, 1942).

*Method.* A drop of ether solution of phosphatide is placed upon a clean microscope slide. After evaporation of the ether, the thin waxy film is pressed beneath a coverslip and the preparation mounted on the stage of the dark-field microscope. The sharply defined edge of the P-lipid film is focussed under the high-dry objective and timed observations are made from the moment contact is established with the watery solution run in by capillarity under the margin of the coverslip. Oil-, or water-, immersion objectives are used for the finer details of microscopic observation.

*Cephalin data.* Pure cephalin (phosphatidyl ethanolamine), under the dark-field microscope, presents a greenish, very faintly hazy film, devoid of significant granulation or other optical appearances. The edge (air-phosphatide interface) is sharply defined but not highly refractile (*cf.* lecithin).

*Distilled water.* Immediately upon contact with water, the edge of the cephalin film shows an initial expansion toward the watery phase. In this, as in other cases of formation of very thin (?) molecularly expanded) films, the appearance of a slight hazy (ultramicroscopic) granulation is noteworthy. After about 30 sec. (average) the still well defined edge develops typical myelin forms. These vary in size and shape, some being cylindrical (usually curved) or pyriform, but they alter and tend to subdivide into spherical forms. The term "vesicle" will henceforth be used with particular reference to the tiny spheroidal figures, which evince a tendency to become discrete and free-floating, although at first they often retain filamentous attachments. A 5-10 min. "delay period" (comparable to that previously described for lecithin films) is occupied with the production of more and more myelin forms and minor changes in the size and shape of the figures.

The "secondary expansion" phenomenon (*cf.* lecithin) is the subsequent more vigorous outflow of the myelin forms into the watery phase. The pulling away of phospholipid fragments of varying sizes with numerous elongating filaments resembles the stretching behavior of taffy, treacle, honey and other grossly viscous materials. Most noteworthy, however, are the tiny (average: 5-25 *micra*) "vesicles." These later develop clearly defined granules, apparently in their interior since they soon begin to oscillate with Brownian movement. Close observation suggests that the granules originate from the envelope or "membrane" of the vesicle, and their gyratory movements are often restricted to the proximity of the surface of the vesicle.

In size, appearance and behavior, these vesicles resulting from the peculiar myelin figure formations from pure cephalin-water films are minutely similar to the "vesicular alterations" previously described for platelets, megakaryocytes and thrombocytes. While the persistence of the vesicular forms for several hours is noteworthy, there is an increasing degree of further breakdown coincident with the gradual appearance of free refractile granules vigorously oscillating (Brownian movement) in the watery phase. These granules are strikingly similar to the *chylomicrons* (hemoconia or "blood dust") of plasma or serum, which have long been regarded as lipoidal in nature.

*Calcium salt.* A weak solution of calcium salt, obtained by diluting 5 parts of N/7  $\text{CaCl}_2$  with 142 parts of water (*cf.* Ca:Na mixtures), gives many appearances comparable with the foregoing (water effect) and some additional findings which resemble the actions of stronger  $\text{CaCl}_2$  solutions.

Immediately on contact, a marked deposition of granules (with a 'silvery' diffraction) starts at the edge of the cephalin film and extends inwards (calcium effect). At the same time, the edge itself expands into a narrow clear zone which quickly breaks up into "vesicular" figures. Within three or four minutes, the more cylindrical (and similar) myelin forms begin to develop and these divide and bud off further vesicles over a period of half an hour or longer. The period of "secondary expansion" starts some minutes after the first formation of the larger myelin forms and soon becomes a vigorous outflow, with detachment of larger and smaller cephalin fragments and filaments. These fragments contain the calcium-deposited granules, in contrast to the earlier myelin forms, which are optically clear for the first 5-10 min. Later, the myelin forms, especially the vesicles, acquire the free oscillating granules previously noted. This may be preceded by an appearance best described as 'shimmering in concentric layers,' faintly visible with the highest powers of the microscope. Eventually the Ca-deposited granules in the outflowing cephalin fragments also begin to oscillate and may be liberated to become indistinguishable from the ordinary "chylomicrons."

N/7  $\text{CaCl}_2$  shows only the phenomenon of granular deposition in the phospholipid. This commences immediately on contact and penetrates deeper and deeper, in devious pathways, to an extent which seems to be determined chiefly by the thickness of the cephalin film. The progressive slowing of the penetration rate suggests that the altered phospholipid imposes a barrier to the inward

diffusion of the calcium salt solution. Within two or three minutes from the start, there is a very narrow clear marginal zone which does not form any myelin figures. The linear edge, after several minutes, breaks down into a series of fine granules which coalesce to become larger and fewer and similar new granules appear in the clear zone. Later, the granulation in the cephalin itself becomes coarser and more refractile and the highest powers of the microscope reveal a globoid (or elongated) outlined appearance up to 1 or 2 *micra* in diameter.

The subsequent addition of M/7 NaCl often leads to an enlargement of the granules and clearing of much of the intervening background, but myelin figures fail to appear.

It is clear from these and other observations with intermediary concentrations of  $\text{CaCl}_2$  that the specific effect of the Ca-ion is to penetrate the cephalin film and cause a deposit of "fixed" granules as seen under the dark-field microscope, corresponding to a cloudy opacity visible to the naked eye inspection of the film. The suppression of myelin figure formation in the stronger  $\text{CaCl}_2$  solutions indicates that calcium can prevent the tendency for cephalin to emulsify in water.

It is significant that "physiological" concentrations of  $\text{CaCl}_2$  are not sufficient to check myelin figure, vesicle, and chylomicron formation, but merely exert a minor delaying and modifying influence. Some further data on the role of Ca will be noted in the observations with Na:Ca mixtures.

*Sodium salt.* N/7 NaCl solution considerably modifies the myelin figure formation from cephalin films. Within half a minute of contact, the phospholipid margin shows a vesiculation and often a slight, perhaps reduplicated, retraction of the edge, leaving several layers of scattered clear vesicles. These vesicles range from 5-50 *micra* in diameter, but often coalesce into larger objects, frequently lentiform when in the free margin. An optical peculiarity is the much feebler refraction of the outer rim of the vesicles and this edge may expand actually to go into solution, usually partially and with a tendency to re-form. On casual glance, the cephalin edge has a curious serrated appearance. After a few minutes, the serrated edge commences a slow expansion into the watery phase and more and more of the vesicle walls go into solution. There is no appearance of granules either in the watery or in the phospholipid phase. Thus, in NaCl solution of "physiological" strength, cephalin shows a very limited and modified myelin figure formation, the end result of which seems to be a partial solution (vesicles disappear) and emulsification (vesicles persist) of the cephalin in the salt solution. The NaCl does not penetrate into the phospholipid. N/7  $\text{CaCl}_2$  solution, subsequently introduced, causes new crops of vesicles to appear, with retreat of the edge of the cephalin. In the vesicles, strands and filaments left behind during this process, the usual type of calcium-granule appears. On reaching an isolated vesicle, the  $\text{CaCl}_2$  causes the outline to break up into a string of granules which coalesce, etc., as in the marginal zone granules described for N/7  $\text{CaCl}_2$ .

A mixture of equal volumes of N/7 NaCl and N/7  $\text{CaCl}_2$  is "unphysiological" but does present an opportunity for any reciprocal antagonism of approximately ionic equivalents of  $\text{Na}^+$  and  $\text{Ca}^{++}$ . The immediate effect is that of the

calcium (see N/7  $\text{CaCl}_2$ ), and the later picture of granulation of a narrow marginal zone is also like that of the  $\text{CaCl}_2$  alone, and quite suppresses the  $\text{NaCl}$  effect.

A mixture of Na:Ca made by adding 0.5 cc. N/7  $\text{CaCl}_2$  to 14.2 cc. N/7  $\text{NaCl}$  (i.e., "physiological" with respect to ion balance and osmotic strength) gives a very weak Ca effect (like the diluted  $\text{CaCl}_2$ ) and the typical  $\text{NaCl}$  effect (like N/7  $\text{NaCl}$ ), which preponderates.

A mixture of 1 cc. each of N/7  $\text{NaCl}$  and N/7  $\text{CaCl}_2$  in 100 cc. of water (i.e., much diluted) behaves like distilled water, except possibly for some lessening in the formation of chylomicrons.

*Alkali.* The addition of a drop of N/10  $\text{NaOH}$  to 5 cc. of (a) N/7  $\text{NaCl}$ , (b) the 142:5 Na:Ca mixture did not modify their respective effects.

*Data on other phospholipids.* The observations on *cephalin* films have brought out two main points, viz. (1) distilled water, alone, causes active myelin figure formation, (2) strong  $\text{CaCl}_2$  solutions produce a granular deposition penetrating into the phospholipid and interfering with its hydroscopic dispersion in the watery phase. A third point is the retarding influence on myelin figure formation of  $\text{NaCl}$  solutions of "physiological" osmotic strength. Comparative studies on the first two points have been made on films of lecithin (commercial) and Folch's brain phosphatides, which may be identified as (I) phosphatidyl serine, (II) an incompletely identified cephalin fraction, and (III) an inositol-containing phosphatide (9). The myelin forms from *lecithin* are very much larger and more refractile than the forms noted for cephalin. The penetration and granular deposition by calcium salt are similar but (*cf.* 8) myelin figure formation, though retarded, occurs in solutions as strong as M/20  $\text{CaCl}_2$ . M/7  $\text{NaCl}$  solution behaves much like distilled water. *Phosphatides I and II*, in distilled water, give myelin forms, including "vesicles," considerably larger than with cephalin. The *inositol-phosphatide* (III) behaves differently, the material going directly into colloidal solution in the form of "chylomicra." In gross appearance, III is a powder, rather than a waxy material like the other P-lipids. N/7  $\text{CaCl}_2$  causes a granular deposition in I, II, and the colloidal solution of III. A more detailed study of these other phospholipids is not included at this time because the data obtained are sufficient to suggest that we must look chiefly to the *cephalin* for appearances upon which to base an analogy with the described alterations of platelets and other formed elements. Owing to anticipated technical difficulties of photomicrography of the minutiae of altering cephalin films, we append only the illustration of the easily photographed larger "myelin forms" of *lecithin* (Fig. 12). These do serve to demonstrate the general features of the phenomena under consideration.

#### DISCUSSION

These experimental data demonstrate the applications of a novel method of investigation and warrant the drawing of some far-reaching conclusions. The reproduction, in artificial water-phospholipid films, of the essential phenomena of surface alterations occurring, *in vitro*, from several formed elements of blood

and bone-marrow, indicates the fundamental nature of these physiological processes, which are amongst the most primitive of cell (protoplasmic) functions. The observations suggest that cephalin is outstanding, amongst the phospholipids studied, for the very close correspondence of its "myelin forms" to the surface alterations of cell protoplasm. The ubiquitous occurrence of phospholipids in cellular material is too well known to require comment, but it may be recalled that cephalin constitutes nearly two-thirds of the total phospholipids isolated from platelets and erythrocyte stroma ("ghosts") (10). It is evident from the data that specific ions, especially  $\text{Ca}^{++}$ , can modify myelin figure formation and related changes involved in the mixing of phospholipids with water. The need for further elucidation of the problem is shown by the stubborn fact that it is distilled water which induces changes in cephalin films, that are most closely similar to the protoplasmic alterations, although the latter normally occur under complicated conditions with respect to osmotic strength of solution, specific ion equilibria, and adsorbed substances (proteins, cholesterol, etc.) (8) which influence "membrane permeability." Persistent differences between simple artificial systems and the complex behavior of protoplasm are to be expected. Evidence of similarities, however, constitutes an important advance toward the understanding of the surface properties of protoplasmic membranes.

#### SUMMARY

A comparison of the dark-field microscopical appearances of (a) water-phospholipid (cephalin, lecithin, and similar) films and (b) in vitro surface alterations of platelets, thrombocytes, megakaryocytes and certain erythrocytes, indicates the fundamental importance of "myelin figures" and related changes at water-lipid interfaces for the explanation of these primitive protoplasmic "membrane" phenomena. The most important factor is the hydrotropism of the lipoidal material. Individual differences between the lipids studied point to a special significance of phosphatidyl ethanolamine (cephalin) in the natural formed elements. The water effect is undoubtedly modified by the osmotic strength and specific ionic properties of dissolved electrolytes (especially  $\text{Ca}^{++}$ ) and probably also by cholesterol, proteins and other materials entering into the formation of cell "membranes." The full significance of these modifications remains to be elucidated.

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### EXPLANATION OF PLATE 3

Dark-field photomicrographs. (Oil-immersion lens)

Fig. 1. "Stellate" forms of platelets in citrated rabbit blood plasma.  
Fig. 2. Recalcified rabbit plasma, showing 1. vesicular alterations of blood platelets, 2. fibrin threads.  
Fig. 3. Coagulating drop of human blood, showing 1. vesicular alterations of blood platelets (fine circles mostly in close relation to granular "body" of platelets), 2. erythrocytes (large refractile circles), 3. granular leucocyte (with dark nucleus faintly discernible), 4. a few fibrin needles.  
Fig. 4. Saponin lysis of platelets in citrated rabbit plasma.

### EXPLANATION OF PLATE 4

Dark-field photomicrographs. (Oil-immersion lens)

Fig. 5. Megakaryocyte from rabbit bone-marrow, mounted in serum (autologous), showing granule-containing vesicular excrescences at periphery.  
Fig. 6. Megakaryocyte from rabbit bone-marrow, recalcified after citration, showing vesicles at periphery.  
Fig. 7. Disintegrated thrombocyte of frog blood, showing vesicles. A large fibrin thread extends through the body of the thrombocyte.  
Fig. 8. Recalcified citrated blood of fresh-water sun fish, showing 1. numerous nucleated red cells, 2. disintegrated thrombocytes (near center) with vesicles and granular material, much of the latter scattered by the explosive disruption of the thrombocytes

### EXPLANATION OF PLATE 5

Dark-field photomicrographs. (9-11 Oil-immersion lens)

Fig. 9. Primitive erythroblasts of rabbit embryo (heart-blood), showing stromatolytic filaments.  
Fig. 10. Erythroblast of tadpole, showing numerous filamentous stromatolytic forms.  
Fig. 11. Human erythrocytes, showing contact development of envelope (membrane) filaments.  
Fig. 12 (a) (High-dry lens) Edge of film of lecithin, showing smooth, refractile air-phospholipid interface.  
(b) Same edge, showing "myelin forms" produced by contact with distilled water.

PLATE 3

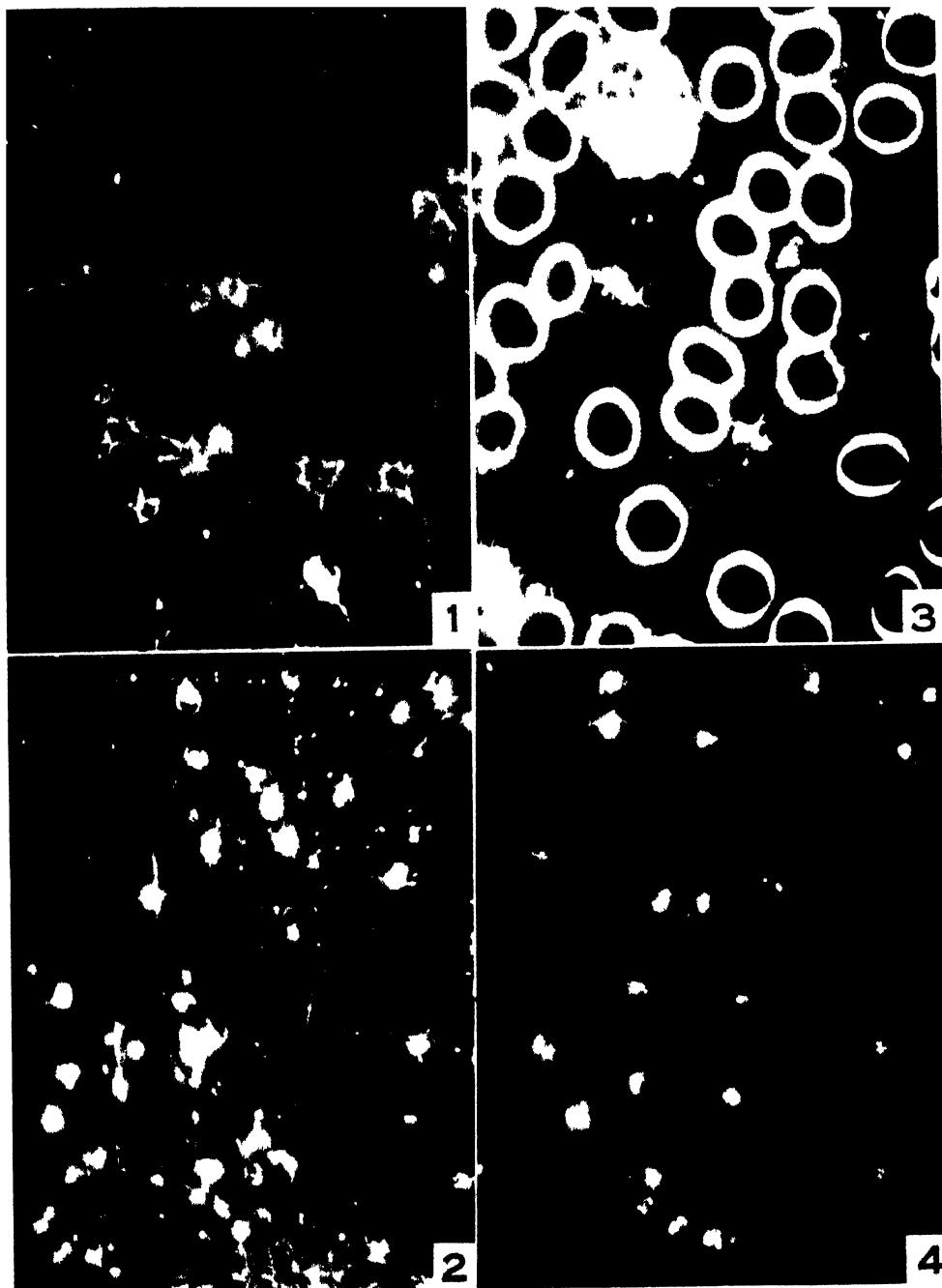




PLATE 4

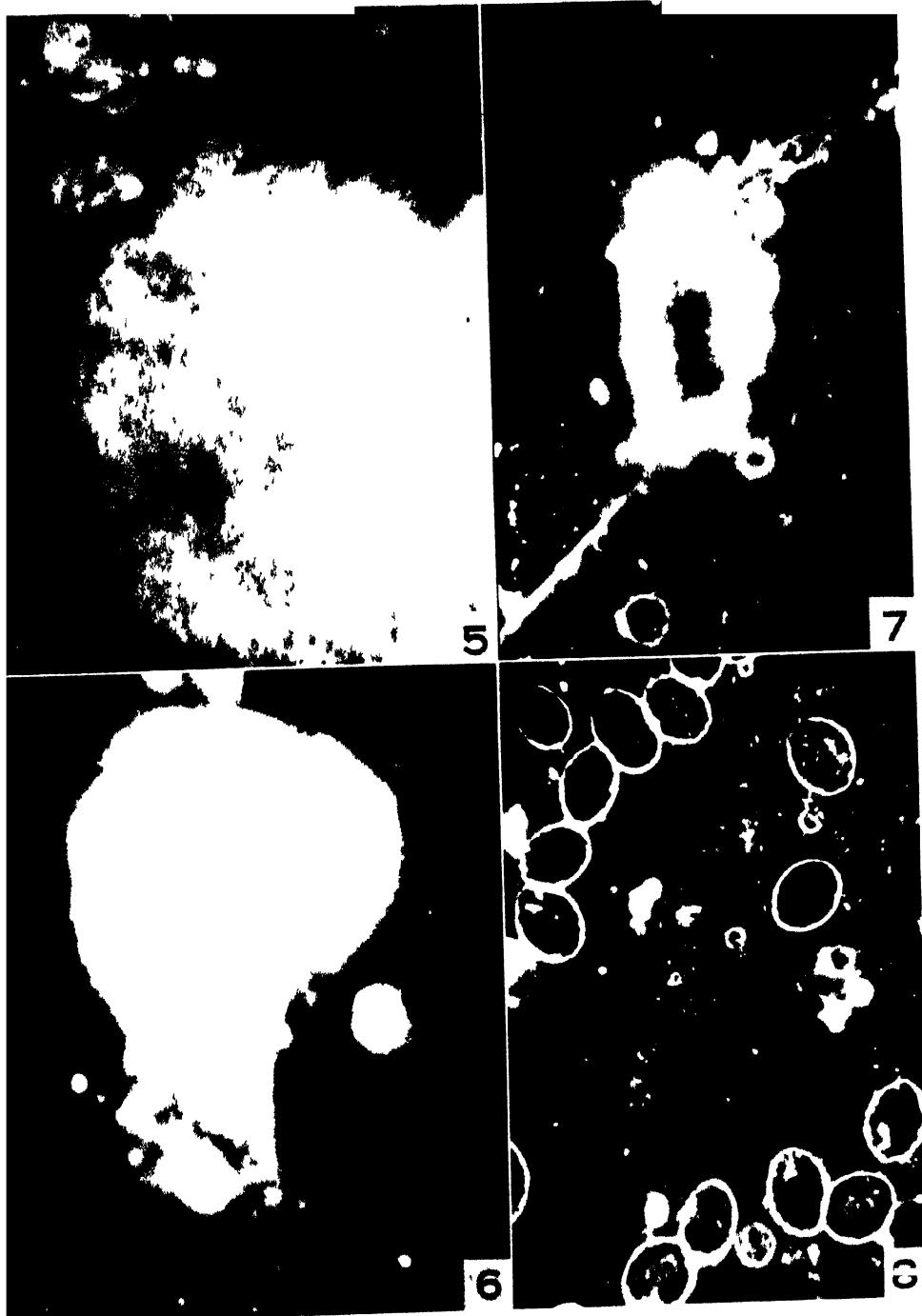
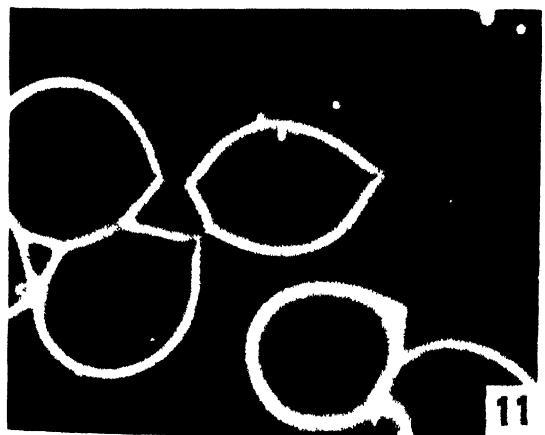
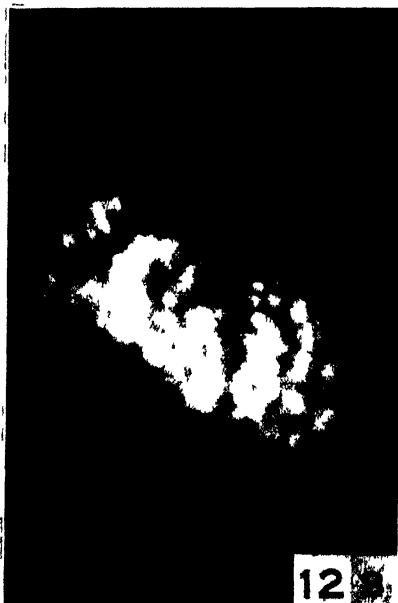
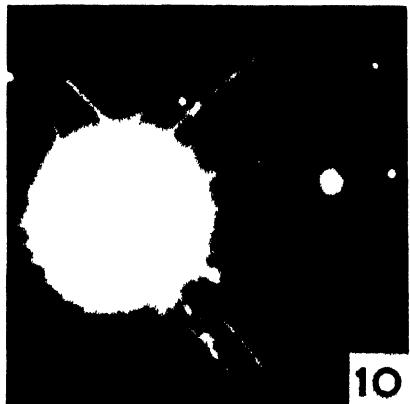
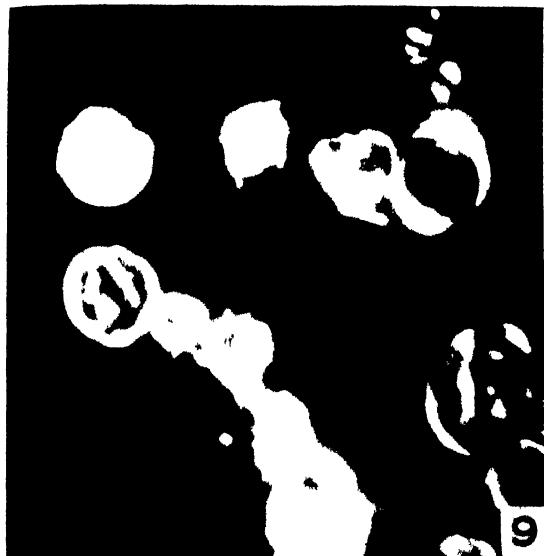




PLATE 5





# REGENERATION OF LYMPHATIC CHANNELS FOLLOWING ABLATION OF LYMPH NODES

BY RUSSELL L. HOLMAN

*From the Department of Pathology, University of North Carolina, Chapel Hill,  
and the Department of Pathology, Watts Hospital, Durham, N. C.*

## PLATE 6

### INTRODUCTION

One of the problems arising in connection with some recent studies on the popliteal lymph nodes of dogs (5, 6) is the manner in which lymph flow from the foot is re-established after the popliteal lymph node is excised. The presentation of this problem is divided into three parts: first, a detailed description of the methods used; second, a brief presentation of the essential findings; and third, a necessarily vague discussion of some of the possible applications and implications of these findings to problems encountered in human pathology.

The problem is not a new one. Regeneration of lymphatic channels has been studied by different methods, in different anatomical regions, in different species of experimental animals, for varying time intervals, by numerous workers (1, 2, 3, 7, 8, 9, 11)—ofttimes with specific questions in mind. And as usually happens in such diversified experiments, some differences of opinion result. We need not concern ourselves with a review of all these experiments. Suffice it to state that the experiments here reported agree in the main with previously published ones using similar methods, in similar anatomical regions in dogs. In the experiments to be reported it is believed that some minor refinements in technique have been introduced, and that one new finding is presented.

### METHODS

Before taking up the actual details of a typical experiment, some general remarks about the methods are in order. The most satisfactory method for studying the lymphatic system, under both normal and pathological conditions, is the injection method. Drinker (2), MacMaster (7), and others have shown that, for all practical purposes, the intracutaneous and subcutaneous injection of particles of colloidal size amounts to an intralymphatic injection. The most satisfactory injection substances are dye-stuffs. India ink, graphite, etc. (suspensoids rather than true colloids), while possessing the distinct advantage of not being washed out and thus yielding suitable preparations for microscopic study and illustration, have not been satisfactory in my hands as the particles do not readily enter the lymphatic channels when injected into the tissue spaces; and intralymphatic injection of these suspensoids is tedious and yields uncertain results.

Numerous dye-stuffs were tried in preliminary experiments. Red dyes were abandoned because of their confusion with blood vessels. Some dye-stuffs

proved toxic when injected in large amounts, others were discarded because of solubility difficulties, and still others were useless because they were reduced to the colorless or "leuko" form when they were injected into the body. Three dyes have proved satisfactory—pontamine blue, trypan blue, and nigrosine. Possibly the best of these is pontamine blue.

If the dye is dissolved in water or in a saline solution—even in amounts to yield isotonic solutions (in my experience this consideration is not important, for the buffers in the circulating fluids readily adjust any minor hypotonicity or hypertonicity)—it diffuses out into the tissues within a few minutes after injection and increasingly blurs the outlines of the lymphatics, thus rendering dissection and diagramming of these channels difficult, if not impossible (Fig. 1). If, however homologous serum is used as the solvent, the dye remains sharply confined to the lymphatic channels for several hours or until it has been washed out by the flow of lymph (Fig. 2).

The injections of serum-dye solution were the same in both the control and the experimental groups. Since the animals were sacrificed shortly thereafter, no precautions were taken in regard to sterility. A 1 to 2% solution of the dye was injected in all instances, and all three dye-stuffs mentioned are soluble to this extent in serum at room temperature.

Before the animal was sacrificed it was bled about 20 cc. from the jugular vein, and the serum obtained from this was used to make a 1 to 2% solution of the dye-stuff. One cc. of this serum-dye solution was then injected into the loose subcutaneous tissue between each of the toes and into the denser subcutaneous tissue on the under surface (sole) of the foot just distal to the foot pad, at least four injections being made into each extremity examined. The dog was then allowed up to run around or the foot was massaged for several minutes in order to stimulate lymph flow and transport of the dye through all the main trunks. In all instances before sacrifice was completed, while the dog was under light ether anaesthesia, an incision was made over the femoral vessels below the inguinal ligament, and the main lymphatic trunks in this region were examined. In all instances in the control group a minute or two of exercise or massage sufficed to outline the lymphatic trunks in the femoral region with dye. In fact, with the animal under anaesthesia, with the skin reflected back and the lymphatic trunks exposed, almost immediately upon massage of the injected foot, the trunks throughout their entire course, including the whole of the popliteal lymph node, became beautifully outlined with dye. Dye can usually be demonstrated in the circulating blood in less than a minute after starting the massage.

If a ligature is placed about the trunks below Poupart's ligament, these trunks distend in a remarkable fashion—often reaching 2 to 3 mm. in diameter—with beading at the sites of the valves. With experience this beaded appearance of the distended lymphatic trunk gets to be quite characteristic, and the lymphatics can be identified with certainty even in their natural unstained condition. If distention and beading do not occur when a ligature is placed about the trunks, one can be certain that a collateral channel is relieving the pressure that would ordinarily be built up by this procedure. Emphasis is laid upon these points,

for many conflicting results can be obtained unless special attention is paid to them.

After the foot was injected and massaged or exercised, and after the trunks in the thigh were ligated and a few minutes were allowed for the pressure thus built up to open up potential channels, sacrifice of the animal with ether was completed and the skin over the entire leg was dissected away. The lymphatic trunks outlined by this procedure were then drawn in on diagrams of the medial and lateral aspects of the leg and any abnormal findings noted. In the control group of animals this constituted the entire procedure.

In the experimental group, at the time of removal of the popliteal lymph node, strict aseptic precautions were observed. In some instances attempts were made to tie off all the severed afferent and efferent lymphatic trunks as well as blood vessels; in other extremities the nodes were removed rapidly with attention being paid only to hemostasis. In all instances the wounds were closed by a layer of sutures through the subcutaneous tissue and another layer through the skin. Attempts at bandaging were not too successful and were soon abandoned. In several instances the dog pulled the wound open and in a few of these slight infections occurred in the depths of the wound. Almost daily notes were kept on the wounds until they were completely covered over with epidermis, 6 to 23 days after the operation. These notes have been carefully checked over, but since there was no correlation between the opening of the wound and healing by first intention or between the presence and absence of infection and the pattern of lymphatic regeneration, these factors have been disregarded in the table to be presented.

Following the operation, usually some edema—sometimes quite noticeable and capable of marked pitting—was noted about the operative wound and especially in the soft tissues about the Achilles tendon. This edema did not become noticeable before the end of the second day, usually reached a maximum by the 4th day, was subsiding by the 5th day, and had practically disappeared in all instances by the 8th or 9th day. Some oozing of lymph from between the skin sutures was usual. I have the definite impression—but not sufficient accurate data to prove it—that, in those cases in which attempts were made to ligate all the severed ends of the afferent and efferent lymphatic trunks, induration and gaping with opening of the wound occurred more frequently, and that the total time required for complete skin healing in these cases was a little longer. This point should be checked more thoroughly.

#### EXPERIMENTAL OBSERVATIONS

The first problem was to establish "normal" for the technique used. The control group, from which "normal" was defined, consisted of 25 extremities in 15 normal adult dogs. Invariably the major trunks coursed over the dorsum of the foot around the *lateral* aspect of the leg (on either side of the external saphenous vein) to enter the inferior pole of the popliteal lymph node. The efferent channels of this node emerged from the superior pole to follow the femoral and

iliac veins to enter the iliac nodes. In only a rare instance did a small branch go to the pubo-inguinal node (Fig. 3).

When the same injection technique was applied to the experimental group (54 extremities in 34 adult dogs at intervals varying from 6 hours to 15 months after extirpation of the popliteal lymph node) the following results were obtained (Table I):

The ten experiments failed to show any gross evidence of regeneration during the first 6 days. In no instance did the lymphatic trunks in the thigh become outlined by the dye injected in the foot; instead dye-stained lymph oozed (in some of the earliest experiments it actually poured) from the severed ends of the afferent channels.

After the 7th day, lymph flow from the foot had invariably become established. In the majority of instances this occurred by a combination of "sprouting across the gap" and the development of a "superficial skin plexus." This latter probably represents an anastomosis between wound sprouts and cutaneous channels,

TABLE I

*Re-establishment of Lymph Drainage from Hind Legs of Dogs after Excision of the Popliteal Lymph Node*

POST-OPERATIVE INTERVAL (days)	NUMBER OF EXTREMITIES	SUPERFICIAL SKIN PLEXUS	MEDIAL CHANNEL	REMARKS
0-7	10	0	0	Edema, oozing, induration opening of wound Thrombi in 2 cases
7-60	22	20	9	Valves in 3 of 9 medial channels
60-463*	22	14	9	Valves in 5 of 9 medial channels
Totals	54	34	18	41% positive for medial channel
Controls	25	0	0	

\* Only 2 extremities over 190 days

which then enlarged in response to the increased flow. This superficial skin plexus usually drained into the pubo-inguinal node, while the lymph which flowed through the channels that "sprouted across the gap" continued on through the main trunks in the thigh to the iliac nodes. This is the pattern of regeneration usually described, and this pattern has been confirmed in the majority of the experiments reported in this paper.

In 18 cases (33% of the total number, or 41% of the cases in which continuity of lymph flow had become re-established) an unexpected pattern developed. This is the point I wish to emphasize, for in none of the publications dealing with lymphatic regeneration has this pattern been mentioned. In these 18 cases, one or more channels arose from the main trunks at the joint corresponding to the ankle and coursed up along the medial aspect of the leg to rejoin the main trunks in the mid-thigh region (Fig. 4). It will be recalled that in the control group no "medial channels" were encountered. Sometimes sprouts from the region of the operative wound would join these medial channels. Combinations

of "medial channel" and "superficial skin plexus", were not infrequent; and in a few instances these were combined with "sprouting across the gap." In 4 of the 18 instances the severed afferent channels became thrombosed and partially converted into a fibrous cord that extended up to the site of the excised popliteal node. In these cases practically all of the lymph from the foot flowed through the medial channel (or channels).

From the data at hand it is impossible to state whether these "medial channels" are newly formed channels or are merely pre-existing channels which opened up in response to the continued stress and strain following the operation. With time there is a definite tendency for the superficial skin plexus to disappear and for the plexus that "sprouted across the gap" to narrow down to 3 or 4 fairly normal-appearing channels. The medial channel is apparently permanent. In only one of the 34 dogs was there any evidence of new lymph-node formation, and this is inconclusive.

#### DISCUSSION

The points of most significance in these experimental observations are: (1) lymph flow from the foot after being interrupted by excision of the popliteal lymph node invariably re-established itself after a week, and (2) in about  $\frac{1}{2}$  of the cases a point of origin of the effective collateral circulation jumped a considerable distance from the operative site. Does this same pattern of regeneration—which might be called *collaterals arising from the next distal embryonic fold*—occur in other regions of the body and in other species of animals, including man?

The necessarily vague discussion of the possible applications and implications of these findings, which constitutes the third and last part of this paper, is divided into (1) theoretical considerations and (2) practical considerations. On the theoretical side anything which leads to destruction of one or more lymph nodes, or which otherwise breaks the continuity of lymph flow could imitate the conditions of these experiments. Thus tuberculosis, syphilis, Boeck's sarcoid, tularemia, typhoid fever, amyloidosis, Hodgkin's disease, leukemia, lymphosarcoma, and metastatic carcinoma and sarcoma could lead to destruction of the node; and thrombosis, tumor embolism, and the surgeon's knife as well as pressure from without could interrupt lymph flow. Possibly some of the unusual spreads of tumors and infections can be accounted for on the basis of some of the findings presented in this paper. For example, it is possible that pathological fracture of the proximal end of the femur (due to tumor invasion) which sometimes occurs in cases of carcinoma of the breast results from permeation—or more probably embolism—along lymphatic channels which course over the aponeurosis of the external oblique muscle (4, 10). By this route the tumor cells could reach the inguinal ligament and thence via other aberrant channels (for each mass of tumor cells that occludes the lumen of a lymphatic channel again opens up the possibilities created by ablation) over the ileofemoral ligament to the femur. It is plausible that this retrograde extension along lymphatic channels occurs only after the axillary lymph nodes (the "normal" pathway of lymphatic drainage from the breast) have been invaded or destroyed by tumor. The extension

of such tumor emboli to the axillary lymph nodes of the opposite side, to the peritoneum, and to the liver (via lymphatics in the falciform ligament) is more easily understandable. All of these unusual extensions are consistent with the unanticipated finding described in this paper, namely the opening up of new or previously closed lymphatic channels at a considerable distance from the point of ablation. If it should be found that the "pattern of regeneration" suggested above—namely "collaterals arising from the next distal embryonic fold"—is a general biological phenomenon, this knowledge might be useful to the surgeon.

On the more immediately practical side, the methods used in this study might be adapted to outline the nodes to be dissected, thus facilitating identification and insuring more complete removal of the nodes in a given area. If human subcutaneous tissue does not differ from dog subcutaneous tissue, dye injected subcutaneously between the fingers should outline the nodes along the axillary vein and the supraclavicular nodes. If this procedure should prove useful, subsequent experience might indicate further injections to outline other groups of nodes. None of the three dye-stuffs was "toxic" for dogs in the amounts used and there is no reason to believe that the dye injections would "spread" the tumor cells any more than does the normal flow of lymph. It is even conceivable that radioactive or other therapeutic substances could be introduced directly into the nodes by this method.

#### SUMMARY

1. The best method for studying normal lymph flow, also for studying that established under pathological conditions, is the injection method in the living subject. The use of a blue or a black dye is preferable as the contrast between the injected lymphatics and the blood vessels is sharper. Pontamine blue, trypan blue, and nigrosine have been found satisfactory for this purpose.

2. For all practical purposes the intracutaneous, and especially the subcutaneous, injection of these dyes amounts to an intralymphatic injection.

3. When the dye is dissolved in water or in a saline solution, it diffuses out into the tissues within a few minutes thus blurring and obscuring the outlines of the lymphatics; but when homologous serum is used as the solvent, the dye remains sharply confined to the lymphatic channels for several hours, or until it has been washed out by the flow of lymph.

4. When dye dissolved in serum was injected in the loose subcutaneous tissue between the toes of 25 hind feet of 15 normal adult dogs, it was found that the main lymphatic trunks (which are outlined almost immediately by this procedure) invariably course over the dorsum of the foot and around the *lateral* aspect of the leg to enter the inferior pole of the popliteal lymph node.

5. When this same procedure was used in studying 54 hind legs of 34 adult dogs at post-operative intervals of 6 hours to 15 months after excision of the popliteal lymph node, the following results were obtained:

a. Lymph flow was invariably re-established after the 7th day.

b. In the majority of instances the continuity of flow was re-established by "sprouting across the gap," but in 18 cases ( $\frac{1}{3}$  of the total number, or 41%

of the cases over 7 days) an unexpected pattern of regeneration took place. Instead of the normal pattern described above, in these 18 cases one or more channels arose from the main trunks which run over the dorsum of the foot, at the joint corresponding to the ankle, and coursed up along the *medial* aspect of the leg to rejoin the main trunks in the mid-thigh region. This unusual pathway is referred to as the "medial channel."

c. A superficial skin plexus developed in 34 of the 44 cases studied after the 7th day.

d. With time there is a tendency for the skin plexus to disappear, but the "medial channel" is apparently permanent.

6. From the data at hand it cannot be stated whether the "medial channel" represents a newly formed channel or is merely a pre-existing channel which opened up in response to the continued stress and strain following the operation.

7. The possible bearing of this unusual pattern of regeneration, which might be termed "collaterals arising from the next (distal) embryonic fold," upon phenomena found in human pathology is briefly discussed.

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## EXPLANATION OF PLATE 6

Fig. 1. Lymphatics over "wrist" of dog 15 minutes after injecting 1% pontamine blue *in saline* into subcutaneous tissues between toes. Note diffusion of dye into surrounding tissues and blurring of lymphatic channels.

Fig. 2. Lymphatics over "wrist" of dog 60 minutes after injecting 1% pontamine blue in *homologous serum* into subcutaneous tissues between toes. The dye is sharply limited to the lymphatic channels.

Fig. 3. Lateral aspect of right hind leg of a dog with the skin dissected away and the lymphatic channels outlined by dye. This represents the "normal" pathway of lymph drainage from the foot. The popliteal lymph node is seen back of the "knee."

Fig. 4. Medial aspect of right hind leg of dog with the skin dissected away and the lymphatic channels outlined by dye—1 month after excision of the popliteal lymph node. The "medial channel" starts at the "ankle" and is joined by a "sprout" from the popliteal region before it enters the main lymphatic trunks in the thigh to drain into the iliac nodes. The superficial skin plexus starts in the popliteal region and drains into the puboinguinal node from which efferent channels pass to the iliac nodes.

PLATE 6

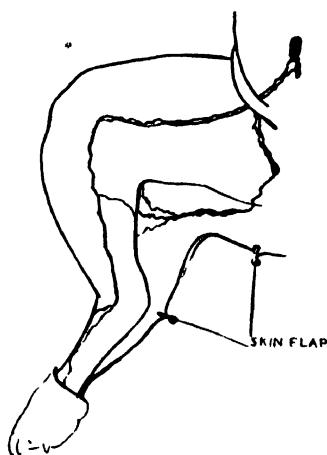


NORMAL



RIGHT LEG (LATERAL ASPECT) 3

MEDIAL CHANNEL AND SKIN PLEXUS AFTER  
EXCISION OF POPLITEAL LYMPH NODE



RIGHT LEG (MEDIAL ASPECT) 4



# THE CYTOLOGICAL REACTION TO INJURY OF THE GLOMERULUS OF THE OPOSSUM KIDNEY (DIDELPHYS VIRGINIANA)<sup>1</sup>

BY WM. DEB. MACNIDER

*The Laboratory of Pharmacology, University of North Carolina*

WITH THREE TEXT FIGURES

A number of years ago a study was commenced in this laboratory of the occurrence of atypical glomeruli in the kidney of the opossum (1). The striking and atypical anatomical feature in these glomeruli consisted in the parietal layer of cells lining Bowman's capsule being formed by a large, discrete order of columnar epithelial cell in place of the flattened, squamous type of cell which is found in this location in the human kidney and also in the kidney of the higher animals such as are usually employed for laboratory observation. Furthermore, in such atypical glomeruli there is a sharp transition in this order of cell from a columnar to a normal flattened type of cell at the point where the parietal layer of cells becomes reflected over the glomerular capillary tufts as the visceral layer of this membrane. On account of this marked difference in structure of numerous glomeruli in the kidney of this phylogenetically old marsupial, the question naturally presented itself as to whether or not in such ancestral animal types there existed such structures in sufficient number to constitute the normal structure of the glomerulus for such forms and if such an observation could be established for the opossum and in addition for other phylogenetically remote types of animals it would then be appropriate to attempt to investigate the method of glomerular function in such kidneys. Lastly, the possibility presented itself that the change in the order of cells lining Bowman's capsule may be an expression of injury to these cells rendered atypical in character either by the influence of the species of animal or by some specifically acting toxic agent for the cells in this location. With these thoughts in mind studies have continued in this laboratory at intervals since the appearance in 1927 of this first paper on the opossum kidney. In addition, microscopic preparations have been studied of the kidneys of the Duck-Bill, *Ornithorhynchus anatinus* (*Paradoxus* Blumenb.), the Spiny Ant-eater, *Echidna aculeata*, and an Australian lizard, *Sphenodon* or *Hatteria punctatum*. These preparations were obtained from the National Museum of Australian Zoology. With two exceptions of a modified order no atypical glomeruli have been observed in the kidneys of these Australian animals comparable to those glomerular modifications which have been described for the opossum kidney.

In the publication previously referred to a report was made on the study of the kidney of 27 opossums. A continuation of the investigation has increased the number of these animals to 58. With the new series of 31 animals urine was obtained either by catheterization or from the bladder immediately after the

<sup>1</sup> This investigation was made possible by a grant from the Josiah Macy Jr. Foundation.

animals were sacrificed. In studies of the urine of the initial group of 27 animals the statement was made that it was free from albumin, glucose and diacetic acid and that it did not contain tube casts. Such observations were made on urine collected in cages which was not fresh and was usually mixed with fecal matter. Similar studies of the urine obtained from the present series of animals either by catheterization or directly from the bladder would indicate that the above statement concerning the character of the urine was inaccurate. In 9 of these 31 animals the urine has contained both finely and coarsely granular casts, erythrocytes and albumin. The kidneys of these 9 animals not only show the presence of atypical glomeruli but an association of other abnormal changes which are to be described.

Upon removal of the kidneys from the animals they were either split in their longitudinal axis and portions of tissue removed from each lateral half, or the kidney was sectioned through its transverse axis in order to obtain a cross section of tissue in this plane. The tissue was fixed in Zenker's and in Bouin's fluid and in a solution of corrosive-acetic, imbedded in paraffin, and sections were stained with eosin and hematoxylin, eosin and methylene blue and with Mallory's connective tissue stain. A study of this material from 31 opossums constituting this new series of animals shows that in 9 of the animals there occurs an atypical order of glomerulus similar to such bodies which have been described in 8 of the 27 animals of the first series. In a total number of 58 opossums comprising the two series, abnormal glomeruli have been encountered in 17 of the animals. The number of such structures in the kidneys of the respective animals has varied. There is no constant numerical relationship between normal and atypical glomeruli. There is a constancy in tissue changes of a chronic inflammatory order between the occurrence of these atypical structures. Whenever they are found there is a variable degree of periglomerular injury characterized by the formation of connective tissue either of a predominately cellular or fibrous order. This is clearly delineated by the use of Mallory's connective tissue stain. In order to ascertain the numerical relationship of normal and abnormal glomeruli in a given kidney the number of glomeruli have been counted in 50 sections which were taken through the lateral half of a longitudinally bisected kidney. These figures have quite naturally varied greatly. The general average for the 9 kidneys so studied which have shown atypical glomeruli has been of the order in a count of 250 glomeruli of 86 abnormal glomeruli to 164 normal structures. The preponderance of normal glomeruli and the association of chronic inflammatory changes with the presence of such abnormal structures would, I feel, eliminate any consideration of glomerular function in the opossum differing from that of the ordinary higher animal such as the monkey, cat and dog. It remains therefore to describe these renal changes in the opossum as a form of glomerulonephritis which so far as I have been able to ascertain has not been previously observed.

A study of tissue from the 17 kidneys presenting evidence of renal injury indicates in the first place that even though the glomerular changes are the most distinctive and characteristic, there also occur modifications in the structure

of the tubular portion of the renal nephron. These changes are very largely localized in the cells of the proximal convoluted segment of the nephron. The cells in such segments are edematous and vacuolated, *a*, Fig. 1,<sup>2</sup> and show poor cell differentiation. The bases of such cells which show less evidence of degeneration are fused together giving a syncytial appearance. Such epithelial changes are not observed in the epithelium lining the ascending limb of Henle's loop which are shown in the figure (Fig. 1) in cross section near the large glomerulus. In this figure is shown one large glomerular unit with its tuft of capillaries, *c*, and embracing the tuft, Bowman's capsule lined by a parietal layer of high, discrete columnar epithelial cells, *b*, which decrease in height as the base of the glomerulus is reached. At this point there is a sharp transition of the order of



FIG. 1

columnar cell which is atypical, to the flattened, squamous type of cell which is not atypical and which is reflected as the visceral layer of epithelium over the capillary tufts. At *d*, is shown the afferent arteriole as it enters the glomerulus to break up into the capillary network, *c*. This network of capillary vessels is definitely abnormal. The lobulations of the network are imperfectly effected, the capillary walls are thickened by a hyaline material and there is an increase in the number of endothelial nuclei which take an intense stain and which in certain glomeruli have been observed to be in the process of multiplication. The size of these atypical glomerular units have varied greatly in both their long and short diameters and the same statement holds true for the maximum and

<sup>2</sup> The figures are camera lucida drawings, Leitz Oc. 2, Obj. 6. Reduced  $\frac{1}{3}$ .

minimum height of the abnormal epithelial cells forming the parietal layer of Bowman's membrane. These changes are indicated in Table I.<sup>3</sup>

The largest glomerulus encountered had a long diameter of  $185 \mu$  and a short, transverse, diameter of  $122.1 \mu$ . The atypical columnar epithelial cells have reached a height of  $25.9 \mu$ , diminishing in this diameter to a squamous order of cell of  $1.5 \mu$ . In addition to the cellular changes which have been described it is to be noted that in part surrounding the glomerulus (Fig. 1) and extending from this area out between the renal tubules there is a variable amount of periglomerular and intertubular connective tissue both in its cellular and fibrous stages. Variations from the maximum order of glomerular change as shown in Fig. 1 are indicated in Figs. 2 and 3, as the abnormal epithelial lining of Bowman's capsule shows a reduction in cell height and finally attains to a squamous order

TABLE I  
*Cell Diameters in  $\mu$*

MEASUREMENTS OF GLOMERULI		MEASUREMENTS OF EPITHELIAL CELLS	
Long diameter	Short diameter	Maximum cell height	Minimum cell height
133.2	129.5	25.9	3.7
129.5	99.9	15.0	7.5
185.0	122.1	21.0	4.5
129.5	103.6	16.0	6.0
129.5	118.4	14.8	3.7
133.2	74.7	18.0	9.0
129.5	111.0	22.5	6.0
129.5	111.0	25.5	7.5
140.6	96.2	10.5	1.5
114.3	114.7	15.0	1.5

of cell. In Fig. 2 the cells of the convoluted segment of the nephron at *a*, show the usual edema and vacuolation which is in striking contrast to the uninjured and evenly staining cells of the ascending limb of Henle's loop, *b*. At *c*, is shown the capillary network of vessels with greatly thickened walls and with an absence of definite capillary lobulation. At *c*, the columnar type of epithelial cell is shown lining Bowman's capsule which in this glomerulus has become considerably reduced in its vertical diameter. At areas in this membrane this tissue is imperfectly differentiated into individual cells and in certain portions is definitely syncytial in its organization. This layer of cells, as the figure indicates, continues to line the neck of the tubule to be followed by a higher order of epithelium, *d*, which is normal for the entire length of the proximal convoluted segment of the renal nephron. In this figure, as was the case with the glomerulus indicated in Fig. 1, the structure is surrounded by connective tissue especially prominent at *f*, and from this periglomerular area connective tissue strands pass between the adjacent tubules. The final transition of the atypical columnar

<sup>3</sup> I desire to thank Professor Edward C. Phiske, of the Department of Anatomy, for his kindness in making the cell measurements.

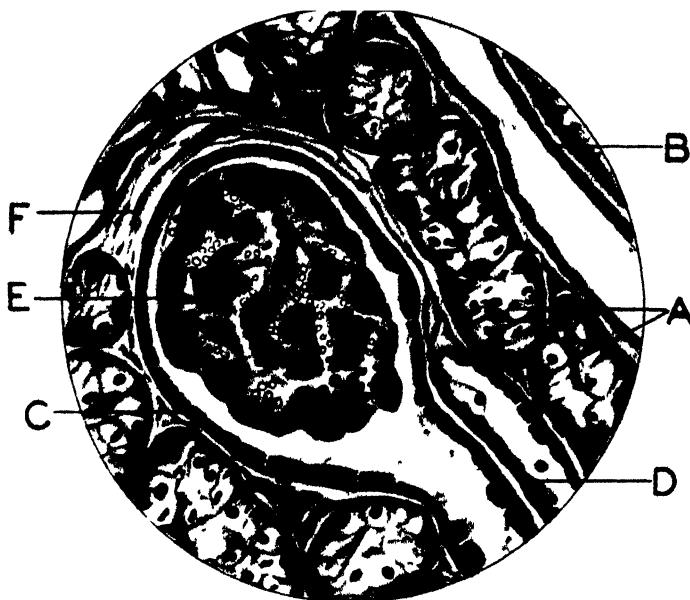


FIG. 2

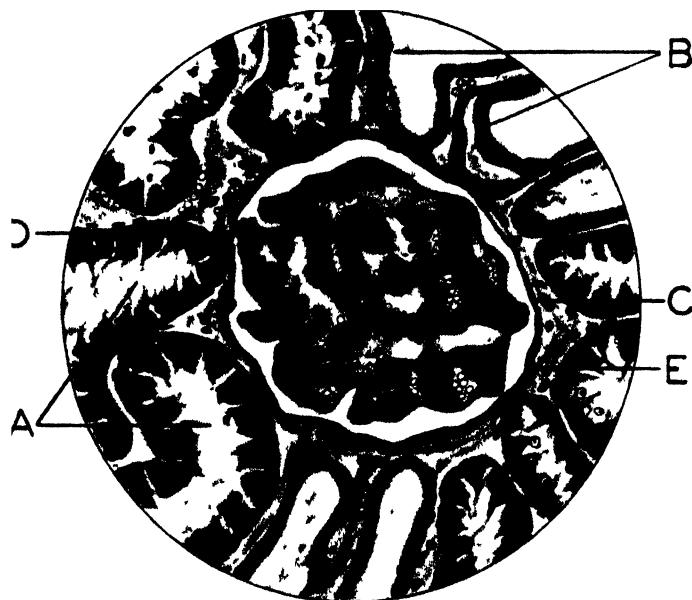


FIG. 3

type of epithelium lining Bowman's capsule to a normal order of flattened squamous type of cell is seen in Fig. 3 at *c*. The entire membrane is formed of cells of this nature, peripheral to which is the capsule, *c*, thickened by connective

tissue. The tuft of glomerular capillaries, *d*, fail to show any definite lobulation which is normal for this structure but appear as vessels with unusually thickened walls containing red blood cells. In the tubules the cells of the loops of Henle, *b*, show no evidence of injury while the epithelium of the convoluted segment of the nephron, *a*, demonstrates the usual edema as has been described for these cells, but less degeneration in the form of cellular vacuolation.

#### DISCUSSION AND CONCLUSIONS

1. The study has been concerned with the description of a reaction of the glomerulus of the opossum kidney to some injurious agent which has been characterized by the lining of the glomerular capsule in a large number of glomeruli in 17 opossum kidneys by a columnar type of epithelial cell which is atypical for this location. Such glomeruli vary greatly in size and the epithelial lining varies from a cell formation with a height of  $25.9\mu$  to the normal squamous type of  $1.5\mu$ . This order of abnormal epithelial lining is confined to the parietal layer of Bowman's membrane. As these cells become reflected over the glomerular tuft of capillaries constituting the visceral layer of the membrane they change abruptly to a normal, flattened type of cell with a thickness of  $1.5\mu$  or less. For this sudden transition in form there is no established explanation. The suggestion is offered, that the flattening out of this layer of cells may in part be due to the pressure exerted on them with each beat of the heart as the capillary loops of the glomerulus become distended with blood and under pressure impinge upon and stretch this visceral layer of the membrane.

2. In the body of this paper reference has been made to a study of microscopic preparations of the kidneys of certain phylogenetically remote animal types. In a few instances sections from the kidney of *Ornithorhynchus anatinus* and from *Hatteria punctatum* have shown glomeruli in which the epithelial cells lining Bowman's capsule appeared unduly prominent. Their vertical diameters were of such an order as to retain them as flattened, squamous types. No cell configuration resembling that which has been described for the opossum kidney has been observed. There is therefore no reason to infer that this order of epithelial structure observed in the opossum kidney is in any measure normal for this marsupial or for certain of the older orders of animals which have been studied.

3. The reasons for considering the glomerular and other changes observed in 17 opossum kidneys as constituting a pathological response on the part of this tissue are to be found in the marked thickening of the walls of the glomerular capillary loops, the periglomerular formation of connective tissue, the extension of this tissue in between the renal tubules, the cytological evidence of injury to the proximal convoluted segment of the renal nephron and finally the association with such structural evidence of renal injury of the appearance in the urine of albumin, erythrocytes and both finely and coarsely granular tube casts.

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## PARATHYROID CYST

By Roy B. McKnight, A.B., M.D.\*

*Charlotte, N. C.*

### PLATE 7 AND ONE TEXT FIGURE

Cystic tumors of the neck constitute a group of lesions which are interesting in their embryologic implications and may present difficult diagnostic problems. Among these are branchiogenic cysts, thyroglossal cysts, hygromas, various cysts of the thyroid gland, sebaceous cysts and other cystic lesions of less frequent occurrence. True cysts of the parathyroid glands, certainly those of sufficient size to be of clinical significance, are almost unheard of.

#### CASE REPORT

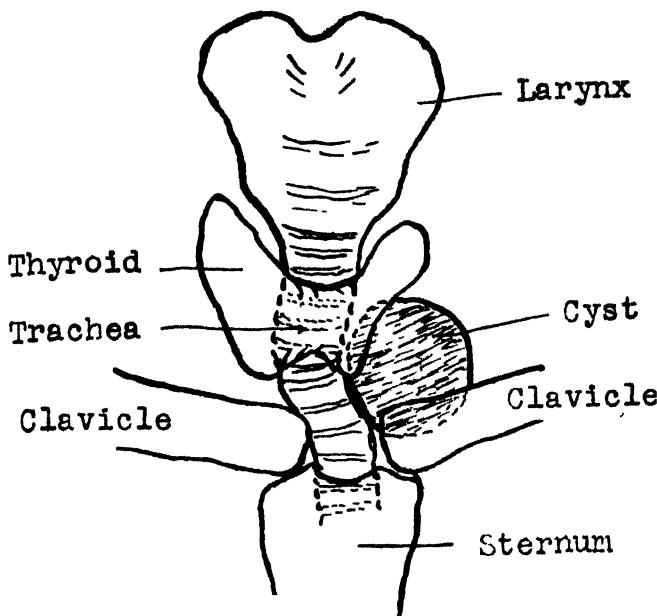
A seventeen year old high school boy complained of hoarseness and moderate dyspnoea on exertion of approximately two months duration. He had known for "sometime" that there was a "lump in the left neck." He had no other complaints. Examination revealed a well developed young man who appeared healthy. There was a hard, round tumor in the region of the lower pole of the left thyroid lobe. It was about the size of a golf ball, slightly movable and had displaced the trachea moderately to the right. Examination was otherwise entirely negative. Blood pressure, pulse, pulse pressure and heart action were all within normal limits. Basal metabolism was not determined since it was obvious the lesion was non-toxic. Blood chemistry studies were not made as there were no clinical indications for them. A clinical diagnosis of non-toxic adenoma, probably fetal adenoma, of the thyroid was made and surgical removal advised. At operation a peculiar type of pearly-colored cyst was found. It was intimately adjacent to, but apparently not a part of, the lateral aspect of the lower left thyroid lobe. The wall of the cyst was inadvertently ruptured just as it was enucleated and contained a pale milky material a little thicker than water. The character of this fluid was such as to give temporary apprehension that some type of dilation of the proximal end of the thoracic duct had been encountered. Such, however, fortunately, was not the case. The thyroid gland was carefully explored and found to be normal in size, color and consistency. Operative diagnosis was deferred pending a pathologic report. The patient made an uneventful recovery.

#### PATHOLOGICAL REPORT

The wall of the cyst, which measures up to 4 mm. in thickness, is composed of parallel bundles of collagenous connective tissue characterized by a paucity of nuclei. Numerous vessels—capillaries and arterioles—are found within the

\* B.A., University of North Carolina, 1914; M.D., Univ. of Pennsylvania, 1920, Assistant Professor of Pharmacology, Univ. of North Carolina, 1922-1924.

wall. Also there are seen several small foci of lymphatic tissue scattered throughout the width of the cyst wall. No epithelial lining is found on the inner surface. Scant, flat endothelial-like cells are seen to form the inner lining of the cyst. Only in one area, throughout various blocks of the wall of the cyst, is there found an epithelial structure, which in its largest diameter measures 3 mm. It is situated in the innermost portion of the wall and protrudes as a small nodule into the lumen of the cyst. The bundles of connective tissue separate and fan out as they approach the nodule, but do not cover the latter at the inner surface of the cyst; the nodule of epithelial cells borders the lumen of the cyst directly.



Schematic drawing showing position of the cyst lying partly subclavicularly on the left and displacing the trachea moderately to the right

The epithelial cells themselves are polygonal in shape and possess a vacuolic cytoplasm with sharply demarcated cell borders and centrally located, vesicular nuclei, which are relatively small in comparison to the cytoplasm. The latter does not possess a visible granulation. The epithelial cells are arranged in clusters and cords, separated from each other merely by a fine reticular network and numerous capillaries which are engorged. The nodule is rather well demarcated from the adjacent connective tissue structures.

No thyroid tissue or other epithelial structures are seen in the wall of the cyst.

The nodule bordering the lumen of the cyst is identical in its histologic structure with that of a parathyroid body, solely composed of so-called water-clear ("wasserhelle") cells. Since the nodule is not separated from the lumen of the cyst by any other structures, it is assumed that the cyst originated from a parathyroid body (4).

## DISCUSSION

Tumors of the parathyroid glands are quite rare and usually consist of the so-called parathyroid adenomas or what has been termed "work hypertrophy." Cysts sufficiently large to give subjective or objective symptoms have not been reported in the English literature, so far as has been possible to ascertain. Goris, quoted by Guy, DaCosta and others, in 1905 made a diagnosis of cystic degeneration of a parathyroid gland on a tumor removed from the neck of a 23 year old male. It consisted of three cysts closely connected, but independent of and not attached to the thyroid gland. Microscopically, it revealed encapsulated colloid and degenerated parathyroid tissue (2). Undoubtedly small cysts discovered on microscopic examination are of not infrequent occurrence. To quote from Herxheimer: "Cysts are very common in parathyroid bodies. Petersen found them six times in one hundred cases; von Verebely in twelve per cent of his cases and similar reports are made by Moller, Erdheim and recently Danisch. I have seen them myself repeatedly. Already Kohn has spoken of their occurrence. It is difficult to state whether the cysts are congenital malformations or due to changes in later life. However, the fact that they increase in number in older individuals point to the probability that the cysts grow in the course of life and only then do they produce an atrophy of the parenchyma of the gland by pressure. The conditions probably are similar to those in the kidney in that minute developmental abnormalities later on grow into larger cysts. The cysts are filled with a light, hardly stainable content in which desquamated fatty epithelial cells or colloid material is found, or they may be filled with a colloid content. In any case they grow by retention of the secretion. They may originate from follicles. Such cysts may grow so large that they may replace almost the entire gland or several of them together may replace the entire organ. These are probably cases of cystic degeneration. As in the kidney one can draw a line from small insignificant single cysts to such formations which deserve the term of cystic parathyroid bodies. An almost complete replacement of glands by cysts has been reported by Schaper in the sheep, Litty in the horse, Rossi in the cow, Aglagna in the dog, Petersen found them in human beings. Harvier speaks of transformation vesiculaire. Erdheim of cystomata of the same formation. Thompson and Harrison distinguish between retention cysts, polycystic degeneration and branchiogenic cysts. There are cysts which are lined by ciliated epithelial cells which have been seen by Edmunds, Kohn, Nicolas and others in the cat, Lusena in the dog, Danisch in his cases in human beings, and it is this type of case which points to developmental abnormality. To this latter type of cyst certainly belong the cysts which are found in juxtaposition to the parathyroid body, although the latter may be partially or completely embedded in its wall, cysts which have been classified as branchiogenic cysts. . . ." (3). Nylander (5) in 1929, reported a "Parathyroid Cyst of the Neck," but neither the periodical nor a translation has been obtained.

It would seem, therefore, that this case is probably the third reported in the literature, that is, of a parathyroid cyst of sufficient clinical significance to give

objective and/or subjective symptoms. It is difficult to classify this cyst. It was filled with a pale milky material which likely contained desquamated cells and maybe some colloid material. The wall was composed of connective tissue and lined with endothelial-like cells, which, of course, were not ciliated. Is it a retention cyst, polycystic degeneration or a "pinched-off" branchiogenic cyst? The fact that some lymphatic tissue is present might suggest its branchiogenic origin. Branchiogenic cysts are lined with epithelium, which under pressure, might become flattened into endothelial-like structures. It is *not* the opinion of the author that this is of branchiogenic origin, but it might be somewhat difficult to disprove it! Apparently the small millimeter-or-so sized cysts reported to be of frequent occurrence are only of academic interest, for it is not recorded that there are any larger than a normal sized parathyroid gland. There is no evidence that they affect parathyroid metabolism.

There was no evidence in this case that parathyroid metabolism was in any way affected. The symptoms were only those of a tumor in the neck and subsequent tracheal pressure manifestations.

#### DEVELOPMENT OF A PARATHYROID CYST

It is hard to give an adequate explanation of why cysts should develop in the parathyroids, or why (certainly large ones) are of such extreme rarity. As stated, it is not infrequent for presumably normal parathyroids to have some follicles containing colloid somewhat similar in appearance to that in the thyroid glands. It is conceivable that one of these follicles might become greatly distended resulting in the cystic parathyroid. The embryological development of the parathyroids is worthy of serious thought as a possible explanation of the cause of this cyst. The superior parathyroids develop as solid outgrowths from the dorso lateral walls of the fourth pharyngeal pouches, and the inferior parathyroids develop as solid outgrowths from the rostral walls of the third pharyngeal pouches. It may be that infrequently one or the other of these solid outgrowths might carry along an extension of the lumen of the pharyngeal pouch. This pouch then might continue to accumulate fluid and so cause a cyst to develop (1).

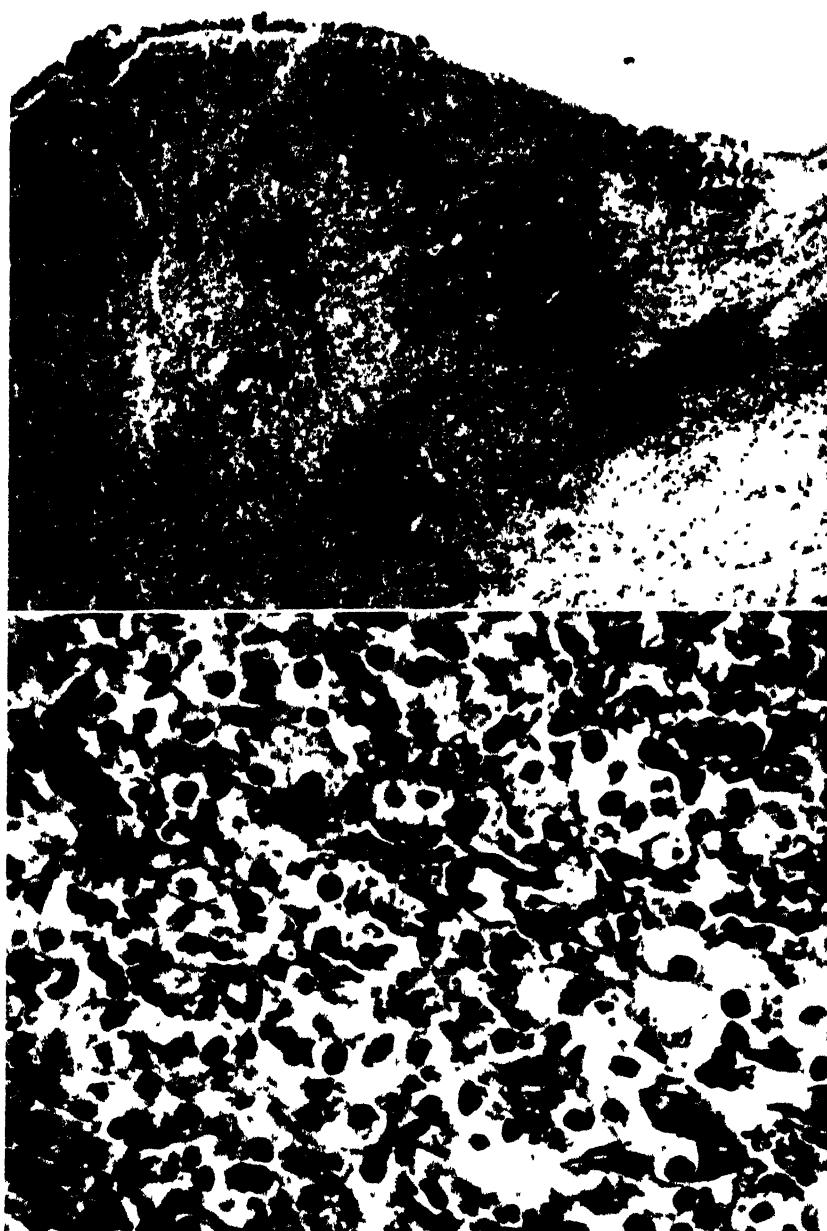
#### CONCLUSIONS

Parathyroid cysts sufficiently large to give objective signs and symptoms are exceedingly rare. Such a case report is presented, together with a discussion of the pathology and possible sources of development. Parathyroid metabolism is not affected.

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PLATE 7



EXPLANATION OF PLATE 7

(Above) Low power microphotograph of the parathyroid gland and a portion (to the right) of the cyst wall. In the wall can be seen a small lymphatic tissue focus. The endothelial-like cells disappear over the parathyroid. There is no evidence of small cyst formation within the gland.

(Below) High power microphotograph showing typical parathyroid cells—the so-called water-clear (wasserhelle) cells. There is no evidence of follicular cyst formation.



# BLOOD VOLUME CHANGES FOLLOWING ACUTE HEMORRHAGE

By A. T. MILLER, JR.

*Department of Physiology, University of North Carolina*

WITH FIVE TEXT FIGURES

The great practical importance of an accurate knowledge of the response of the organism to acute hemorrhage has stimulated a large volume of work in recent years. As a result, much information of fundamental theoretical value has been obtained, leading toward a better understanding of some of the factors concerned in the maintenance of physiological homeostasis.

The immediate crisis in acute hemorrhage is circulatory in origin—the necessity for adjustment of the blood volume to the capacity of the vascular system, so that an adequate circulation of the blood may be maintained. If this is not achieved, the ultimate crisis—tissue anoxia—supervenes.

It must be emphasized that reduction in the size of the vascular bed by vasoconstriction is purely an emergency measure, and is not without attendant disadvantages, involving, as it does, the curtailment of the volume flow of blood through certain regions of the body. Restoration of a normal blood volume is the ultimate compensation, and as such constitutes a logical point of departure in a comprehensive study of the adjustments of the organism to hemorrhage.

The restoration of blood volume following hemorrhage is clearly divisible into two stages: restoration of plasma and restoration of the formed elements. There is general agreement that the restoration of plasma begins shortly after hemorrhage, while that of erythrocytes does not begin for several days. An initial increase in total circulating red cell mass by the discharge of stored cells is still controversial, and apparently depends on such factors as species differences, anesthesia, etc. Little is known about the time course of the restoration of the leukocytes and blood platelets, and experiments on this topic will be reported later.

Most of the early, and some of the recent studies on plasma volume changes after hemorrhage are open to the criticism that these changes were deduced from changes in plasma protein concentration or hematocrit cell volume, not measured directly. These indirect techniques depend for their validity on two assumptions, first that the reference factor (total circulating red cell mass or total circulating plasma protein) remains constant during the test period, and second that a sample of blood drawn from an artery or vein is truly representative of the entire volume of blood. Both of these assumptions are opposed by a considerable body of evidence.

In the human, the spleen is of negligible importance as a red cell storage depot and the total circulating red cell mass remains approximately constant for several days following acute hemorrhage (1), so that hematocrit cell volume changes should be an accurate index of plasma volume changes. It is obvious that only

relative values are obtained in this way and that single determinations may be grossly misleading. In experimental animals the validity of hematocrit cell volume changes as criteria of plasma volume changes is extremely doubtful. Not only does the spleen play a variable role as a red cell reservoir (2, 3), but this factor is even more unpredictable when animals are under the influence of anesthesia (3, 4). There are numerous instances in published work from this and other laboratories (5, 6) in which hematocrit cell volume changes indicated hemoconcentration, in spite of direct evidence of increasing plasma volume.

The magnitude of the error introduced by assuming that the cell-plasma ratio of a sample of blood drawn from an artery or vein is representative of the total volume of circulating blood is uncertain (1, 7, 8) and is doubtless influenced by circulatory disturbances such as are encountered in shock and hemorrhage.

Plasma protein concentration is a valid index of plasma volume following hemorrhage only during the initial phase of hemodilution with protein-poor fluid. The uncertainty (1, 14) regarding the time course of plasma protein restoration makes its use of doubtful accuracy after the first few hours following hemorrhage.

The work to be reported here constitutes a preliminary approach to the broad problem of the compensatory adjustments of the organism to an acute reduction of the blood volume by hemorrhage, and is particularly directed toward a critical evaluation of the commonly used methods for studying alterations in blood volume.

*Methods.* The technique for following plasma volume changes by the dye dilution method has been described in a previous paper (5). Certain refinements of the method necessitated by the rapid changes in plasma volume following hemorrhage will be described in a later paper. Serum protein concentration was determined by the Kjeldahl method in the earlier experiments, and by the falling drop method (9) in the later experiments. Results by the two methods agreed closely. Hematocrit cell volume was determined by centrifugation in rubber-capped Wintrobe tubes for 1 hour at 2500 r.p.m. Heller and Paul's oxalate mixture was used as an anticoagulant. All experiments were performed on trained, unanesthetized dogs after 24 hours fasting with free access to water.

*Results.* The fluid shift following hemorrhage is divisible into two fairly clearly distinguishable phases (see figure 1) which may for convenience be referred to as the rapid and the slow phases, respectively. The rapid phase begins during the hemorrhage itself (if the duration of bleeding is longer than a minute or so) or very shortly thereafter, and is usually completed within an hour. The fluid which enters the circulation during this phase is protein-poor and the fluid shift is self-limited by the resulting plasma protein dilution. The initiating factor in this rapid fluid shift is the fall in capillary blood pressure which reduces the net filtration pressure below the net reabsorption pressure. There are at least two factors concerned in this decline in capillary blood pressure: (a) the initial disparity between the volume of circulating blood and the capacity of the vascular system, and (b) the compensatory vasoconstriction which results in a greater pressure fall along the arterioles. The relative importance of these two factors has not yet received adequate experimental evaluation.

The magnitude of this initial fluid influx during the rapid phase is extremely variable, but is usually sufficient to restore from one-third to one-half of the

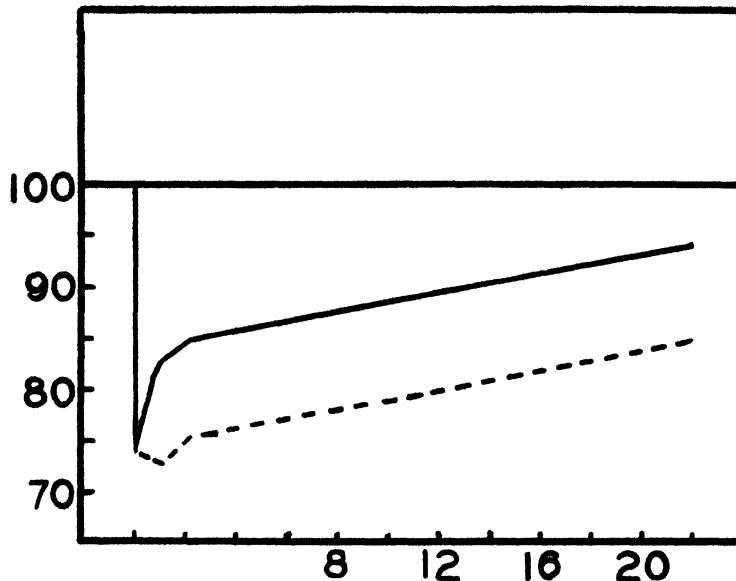


FIGURE 1. Restoration of plasma volume (upper curve) and circulating plasma protein (lower curve) after hemorrhage. Ordinate: plasma volume and total circulating plasma protein in percentage of pre-hemorrhage values; abscissa: time in hours.

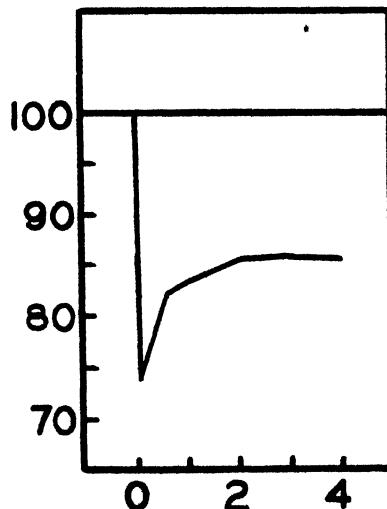


FIGURE 2. Average of 8 experiments, illustrating rapid fluid influx during first hour and negligible fluid gain during next 3 hours. Ordinate: plasma volume in percentage of pre-hemorrhage value; abscissa: time in hours.

plasma water lost in hemorrhage (figure 2). On theoretical grounds, it would be expected that, regardless of the amount of blood lost in hemorrhage, the influx of protein-poor fluid would continue until the lowered colloid osmotic pressure

which resulted would once more reach an equilibrium with the diminished capillary blood pressure. Any further increase in plasma volume would then depend on a mobilization of plasma protein to maintain an adequate colloid osmotic pressure. Actually, the situation is much more complex, although the broad principles outlined seem to be valid. The interaction of a number of dependent variables has still to receive adequate controlled study. A partial listing of these "functions," to use the terminology of L. J. Henderson (10), would include: capillary blood pressure (which in turn is a function of arterial and venous blood pressures, arteriolar vasoconstriction and the size of the capillary bed—i.e., the number and caliber of open capillaries), tissue tension or turgor, colloid osmotic pressure of blood and interstitial fluid and finally an adequate and readily available source of fluid for hemodilution. Some of these factors have received partial experimental study, while others have been almost completely neglected. A comprehensive evaluation of their mutual interaction would be technically difficult but extremely valuable. In the present experiments no such attempt has been made, but rather a critical study of the time course of plasma volume restoration after hemorrhage of standard amount and under as nearly as possible controlled conditions. This was thought to be a necessary preliminary to the more detailed type of study.

The second, or slow phase of fluid influx begins about one hour after hemorrhage and continues for 24 to 72 hours. The restoration of plasma protein proceeds at approximately the same rate as that of fluid (figure 1), and the rate of fluid influx seems to be conditioned by the available reserve of plasma protein or protein building materials. This is well illustrated in figure 3. On March 16, 1944, 22.5 per cent of the blood volume was removed by hemorrhage. The initial serum protein concentration was 6.10 grams per 100 ml., a normal value. Twenty-two hours later, the plasma volume was 114 per cent of the pre-hemorrhage level, representing a total mobilization of fluid equivalent to 36.5 per cent of the initial plasma volume. On November 29, 1944, the serum protein concentration of this same dog had fallen to 4.80 grams per 100 ml., possibly due to protein deficiency in the diet. Twenty-two hours following the removal of 27.5 per cent of the blood volume, the plasma volume had risen to 98 per cent of the pre-hemorrhage value, representing a fluid influx equivalent to 25.5 per cent of the initial plasma volume. Since there is no reason to believe that the supply of fluid available for hemodilution was different in the two experiments, and since the same results were obtained in similar experiments on other dogs, it is a justifiable assumption that the smaller protein reserve, which usually accompanies dietary hypoproteinemia, resulted in a slower rate of protein mobilization, which in turn limited the rate of fluid mobilization. This point could be tested by experiments on animals whose protein reserves had been depleted by plasmapheresis, and such studies are contemplated.

*Discussion.* On the basis of direct determinations of plasma volume by the dye dilution method, the following analysis may be made of the time course of plasma restoration after hemorrhage. Immediately following acute loss of blood, there is a rapid influx of protein-poor fluid, the result of a disturbance in the

balance between capillary filtration and reabsorption pressures. This rapid phase of hemodilution restores one-third to one-half the fluid lost in moderately severe hemorrhage (20 to 30 per cent of the total blood volume) and is complete in about 1 hour. There is no evidence in these experiments of a very rapid hemodilution occurring during the period of hemorrhage. This rapid fluid influx is limited by the resulting protein dilution, and there is little further fluid gain for several hours. With the beginning of protein mobilization 4 to 6 hours after hemorrhage, the slow phase of hemodilution begins and continues for 24 to 72 hours. At this time, the blood volume is approximately normal once more,

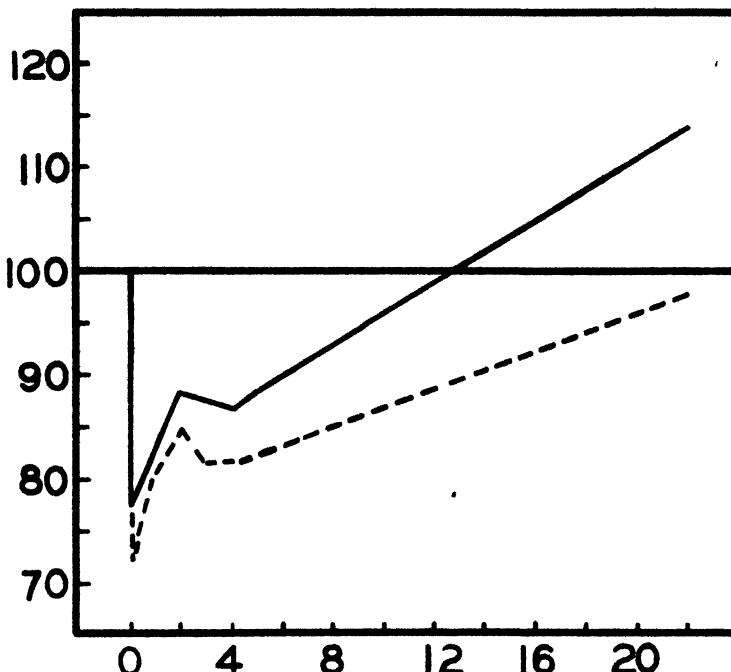


FIGURE 3. Influence of initial plasma protein concentration on rate of fluid influx. Upper curve: plasma volume restoration with normal initial plasma protein concentration; lower curve: plasma volume restoration with low initial plasma protein concentration. Ordinate: plasma volume in percentage of pre-hemorrhage value; abscissa: time in hours.

indicating that the plasma volume increase has been sufficient to compensate for the volume of cells as well as that of plasma lost by hemorrhage. Later, with the gradual restoration of formed elements, there is a reciprocal decrease in plasma volume, maintaining an approximately constant total blood volume.

Another phase of this problem which is now being studied concerns the accuracy of calculations of blood volume changes based on changes in hematocrit cell volume and plasma protein concentration. Figure 4, reproduced from a previous publication (5), illustrates the changes in hematocrit cell volume following a hemorrhage of one-fourth the blood volume in 14 experiments. While the average trend, shown by the interrupted line, is in the direction of hemodilution,

there are numerous examples of apparent hemoconcentration, although simultaneous determination of plasma volume by the dye method indicated hemodilution. It is apparent that the total circulating red cell mass does not remain constant during the 4 hours following acute hemorrhage, and consequently is not a reliable index of blood volume changes. Since changes in plasma or serum protein concentration have been widely used as indications of blood volume changes (see especially 11, 12, 13), simultaneous determinations of plasma

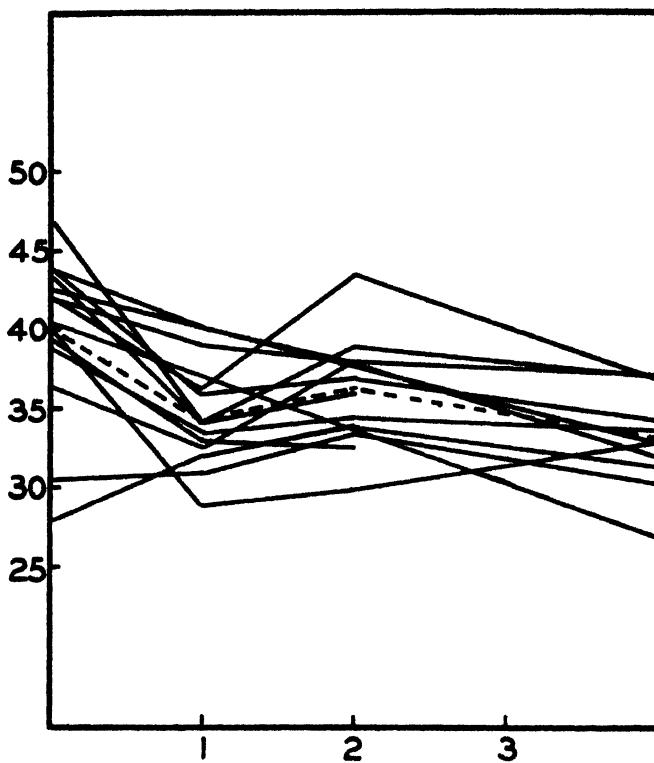


FIGURE 4 Hematocrit cell volume changes following hemorrhage. Ordinate: hemato-crit cell volume in per cent; abscissa: time in hours.

volume and of serum protein concentration were made in a number of experiments. Figure 5, also reproduced from a previous publication (5), illustrates the average changes in total protein, albumin and globulin following hemorrhage of one-fourth the blood volume in 9 experiments. The plasma volume 4 hours after hemorrhage, calculated from protein dilution, was approximately 88 per cent, which is in satisfactory agreement with the value of 86 per cent obtained by direct measurement with the dye method. With the beginning of protein mobilization 4 to 6 hours following hemorrhage, direct measurement of plasma volume is the only accurate procedure. The close agreement between plasma volume changes calculated from protein dilution and measured directly by the

dye method offers additional evidence that protein mobilization is negligible in the first few hours after hemorrhage.

*Summary.* Immediately following acute, sub-lethal hemorrhage there is a rapid influx of protein-poor fluid which restores approximately 40 per cent of the lost plasma. This rapid phase of hemodilution, which is complete in 40 to 60 minutes, is checked by the resulting protein dilution, and little further fluid gain occurs during the next 2 or 3 hours. With the beginning of protein mobilization 4 to 6 hours after hemorrhage there begins the slow phase of hemodilution which

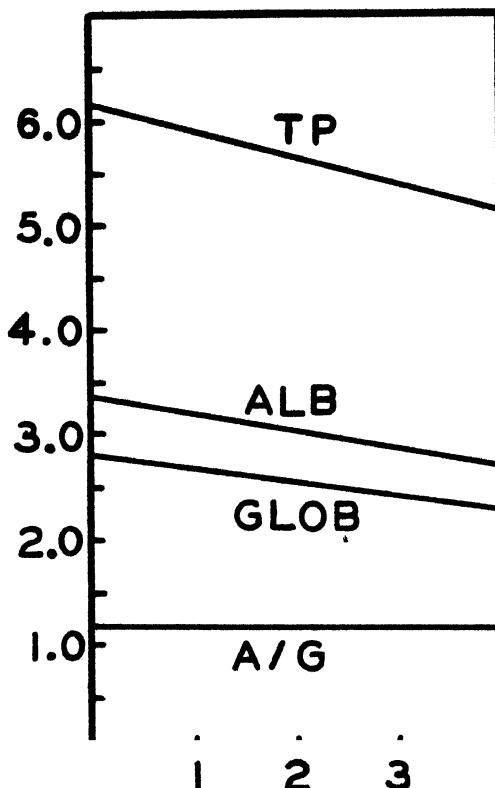


FIGURE 5. Changes in total protein (T.P.), albumin (ALB), globulin (GLOB) and albumin: globulin ratio (A/G) following hemorrhage. Ordinate: grams of protein per 100 ml. of serum; abscissa: time in hours.

lasts 24 to 72 hours and results in a final fluid gain approximately equal to the volume of both cells and plasma lost in hemorrhage. With the beginning of red cell restoration some days later, there is a concomitant decline in plasma volume, so that the total blood volume is maintained at a normal level.

During the first few hours following hemorrhage, the mobilization of plasma protein is negligible, so that protein dilution gives a fairly accurate indication of the degree of hemodilution. On the other hand, changes in hematocrit cell volume frequently give grossly misleading impressions of both the magnitude and direction of blood volume changes.

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# THE RÔLE OF THE FIBROBLAST IN ACUTE INFLAMMATORY REACTIONS WITH REFERENCE TO PHAGOCYTIC EXUDATE CELLS<sup>1</sup>

BY EDWARD C. PLISKE

*Department of Anatomy, School of Medicine, University of North Carolina*

## PLATE 8

The inflammatory reaction is a subject which has engaged the interest of many departments of medical science since early medicine. Its scope is so great that the cytologist, physiologist, immunologist, biochemist and pathologist have united efforts to explain the fundamental nature of the reaction and its significance relative to disease. Menkin (1940) in his monograph "Dynamics of Inflammation" has performed a service of great value by integrating and interpreting the many contributions made by the united efforts mentioned above.

Although the orderly sequence of the cytological aspect of the inflammatory reaction has been recognized since the early work of Beattie (1903), there still remain many cytological problems pertinent to the subject which require elucidation. One such problem concerns the rôle of the fibroblast (fibrocyte) in the inflammatory reaction. Maximow (1905) has maintained that the fibroblast is a highly differentiated cell (end cell) incapable of transformation into other cell types. He has concluded that the fibroblasts play no part, at best a passive rôle, in the cytological sequence with the exception that they divide by mitosis and effect final reparation in the end stages of the inflammatory reaction. Von Möllendorff (1926) on the other hand, attributes considerable embryonic potentialities to the fibroblast in inflammation. His observations, in sharp contrast to those of Maximow, strongly indicate that fibroblasts are stimulated by the inflammatory process to differentiate into free phagocytic exudate cells. In the light of Von Möllendorff's observations, the network of fibroblasts represents a high potential source of phagocytic cells and provides a significant contribution to the cytological picture in inflammatory reactions.

There is a high degree of consonance among investigators regarding the early changes which the inflammatory irritant elicits. Vascular dilatation, transmigration of plasma and large numbers of polymorphonuclear leucocytes from capillaries of the region can be followed with relative ease. However, in the later stages when large numbers of mononuclear leucocytes leave the vessels and rapidly differentiate into macrophages, the intermingling and superimposition of the hematogenous with the histogenous macrophages presents a picture of considerable confusion, especially with respect to the responses of the fibroblasts.

Since much of what is known today regarding the cytological sequence in acute inflammation has been based on connective tissue spread preparations and sec-

<sup>1</sup> I am grateful to Dr. Hal Downey, Department of Anatomy, University of Minnesota School of Medicine, for his guidance in this study. I also wish to thank the Department of Zoology, University of Minnesota, for valuable help with the illustrations.

tioned material taken from the inflammatory site, the examination of these preparations in the present study seemed expedient in order to gain some understanding of the features of the sequence which are in consonance and those which are not. The individual and composite cytological virtues of each technique, therefore, were explored in an effort to better understand the nature of the cellular response, and to lead to, if possible, the development of a more adequate technique which would help to clarify the rôle of the fibroblast in inflammation.

#### MATERIALS AND METHODS

Adult male rabbits were used in this study. All animals were examined periodically for skin lesions during a fifteen day period of isolation. Only animals free of skin lesions were used for experimentation. A stock diet of Purina Chow was supplied *ad libitum* and all animals received fresh drinking water daily. Three times weekly each animal received a diet supplement consisting of carrots, greens and approximately 25 cc. of whole milk.

Typhoid vaccine was selected as the irritant to be used in inducing inflammation. When not in use, the toxoid was kept in sterile vials and under constant refrigeration. All animals were given a standard injection of 1 cc. in the hypodermis of the lower left flank. Immediately following injection, each animal was placed in a clean cage and the hind feet were wrapped with adhesive tape to prevent the animal from scratching the lesion as it developed.

Two groups of animals, 8 in each group, were used for this study as follows:

*Group I.* The animals were injected in quick order and given a number, from 1 to 8, corresponding to the order of their injection. Tissue was taken from the inflammatory site of these animals in the following post-injection periods:

Animal 1	1 hour post-injection
Animal 2	2 hour post-injection
Animal 3	4 hour post-injection
Animal 4	8 hour post-injection
Animal 5	12 hour post-injection
Animal 6	24 hour post-injection
Animal 7.	18 hour post-injection
Animal 8	72 hour post-injection

Sufficient tissue was excised from each animal for several connective tissue spread preparations and for sectioning. The connective tissue spread preparations were fixed in Helly's solution and stained with Weigert's hematoxylin and with methyl-green pyronin. Weigert's stain proved to be of superior cytological value. Tissue taken for sectioning was fixed in Allen's modification of Bouin's. All sections were cut at 5 micra and stained with iron hematoxylin and acid orange G.

*Group II.* The second group of animals were injected in the same manner as described above. Connective tissue smear preparations were attempted on this group at post-injection periods corresponding to those of group I.

Since the management of the smear method is directly related to the problem as a whole, its application will be considered in conjunction with the observations.

OBSERVATIONS OF THE CYTOLOGICAL SEQUENCE, WITH SPECIAL REFERENCE TO  
THE FIBROBLAST, AS EXHIBITED BY CONNECTIVE TISSUE SPREAD PREPARA-  
TIONS AND SECTIONED MATERIAL (1 TO 72 HOURS POST-INJECTION)

*One Hour*

The most prominent features of this early period concerned changes in the capillaries and the development of an edema. The large majority of the capillaries near the site of injection were dilated and engorged with blood cells while a smaller number of capillaries appeared normal and unaffected by the irritant. In every instance the dilated vessels showed changes in the endothelial cells. Irregular bleb-like masses of cytoplasm on the lumen side of the cells could be seen to extend into the lumen. Many of these blebs contained fine neutrally staining filaments, while others appeared entirely clear. The endothelial cells of all dilated vessels appeared swollen especially in the region of the nucleus. Although the vessels were congested with blood cells (chiefly heterophils) no signs of margination were observed.

Prominent fluid-filled spaces were observed between the cellular and inter-cellular constituents of the tissue for several m.m. from the site of injection. Heterophils, red corpuscles and other cellular elements of the peripheral blood filled the spaces in the region immediately adjacent to the site of injection. Surrounding this region, the spaces contained chiefly heterophils. Passage of blood cells to the tissue spaces appeared at this stage to be due to hemorrhage of injured vessels rather than migration through the walls of the vessels.

In general the connective tissue cells of the reacting area exhibited but few obvious changes. The fibroblast net at the site of injection was disrupted by the expanding force of the injection mass and many of the cells appeared pyknotic and degenerate. Peripheral to this point the fibroblasts were greatly attenuated and widely separated by the pressure of the edema. In very favorable sections and spreads delicate, clear cytoplasmic processes of the fibroblasts could be detected spanning the fluid-filled spaces and joining with adjacent cells. Clamatoctyes present in the tissue stained intensely, but otherwise appeared to be normal and inactive. The fibrous elements of the connective tissue presented no notable changes with the exception that they were more widely separated by the serous fluid.

*Two Hours*

All of the capillaries examined in this period were dilated and congested with blood cells. Swollen endothelial cells with prominent darkly staining nuclei were uniformly present in all capillaries. When seen on the flat surface, the cytoplasm of the endothelial cells showed many minute vacuoles. The small bleb-like projections, observed in the first hour, could still be detected between the packed leucocytes. Most of the capillaries exhibited some leucocytes adhering to the roughened lumen side of the endothelial cells.

A moderate increase in edema was evident generally throughout the tissue. The fluid-filled spaces contained from a few heterophils to none at all. Only an occasional red cell could be observed among the heterophils.

No significant changes could be observed in the fibroblast net over the preceding period. At the site of injection a few degenerating fibroblasts were observed still attached to the net; and except for these indications, the exact entrance point of the injection was difficult to locate. The clasmatoctyes adherent to the fibroblast net showed notable changes. Many of these cells had withdrawn their processes and had rounded up. The nuclei stained so intensely that no distinct texture to the nucleus could be discerned. The cytoplasm contained a large number of vacuoles of various sizes. No active signs of phagocytosis could be seen. An occasional cell contained some small granules within some of its vacuoles. Although many of these cells had rounded up, none were seen free in the abundant exudate.

#### *Four Hours*

No notable changes could be observed in the blood vessels over the previous period. A greater number of heterophils appeared adherent to the endothelial cells which suggested an early stage in margination. Passage of these leucocytes through the vessel walls to the tissue edema could not be observed at this time.

During this period a number of endothelial-lined vessels free of blood cells were very prominent. Their large size indicated that they were lymphatics. These vessels were not apparent in the previous periods. The endothelial cells of their walls, although quite prominent, appeared to be less affected by the irritant than the endothelial cells of adjacent capillaries. A granular coagulum mixed with fine threads, of what appeared to be fibrin, formed the only contents of the lumen.

Tissue edema was further increased over the previous periods. A low, firm elevation could be observed on the surface of the skin radiating out about 6 m.m. from the site of injection. The general architecture of the fibroblast net was slightly altered from the preceding period. This alteration consisted primarily of the conformance of the net to form a sparse cellular wall completely or partly around the large lacunae of fluid. The collagenous fibers exhibited no changes with the possible exception that individual fibers showed further separation from one another than in the previous period.

Mitotic figures were rather common in the clasmatoctyes in this period, but there was no apparent increase in the number of cells or their relation to the fibroblast net.

#### *Eight Hours*

All of the vessels of this period showed not only conspicuous margination, but active passage of the heterophils through the endothelium to the tissue exudate. The exudate contained many of these cells especially in the immediate vicinity of the vessels. Emigrant cells revealed no cytological alterations.

No noteworthy changes could be observed in the connective tissue. An increase in edema and an expansion of the reacting area was more apparent on the surface of the skin than by histological examination.

### *Twelve Hours*

Accelerated emigration of heterophils from capillaries characterized this period. Innumerable heterophils pervaded the connective tissue uniformly. Samplings from various sectors of the inflamed area presented the same monotonous picture. Although the number of heterophils was considerable, they did not at this time obliterate the pattern and cytological features of the connective tissue. Many of the capillaries of this period contained small and medium sized lymphocytes as well as an occasional eosinophil. A sparse number of these cells could also be found in the exudate; however, none were actually observed passing through the vessel walls. The lymphocytes exhibited a mild degree of cytoplasmic hypertrophy.

A comparison of the fibroblast net of this period with the previous period showed few significant changes. The clasmacytes, however, wherever observed, showed considerable cytoplasmic hypertrophy. Many of these cells projected free into the exudate attached by a small cytoplasmic process to the net. Mitotic figures were common and several cells in a late telophase were observed. While hypertrophy was the rule with these cells, no phagocytosis on their part could be observed. The highly cellular appearance of the tissue during this period showed a marked contrast to the four hour period where edema and intercellular elements predominated.

No conspicuous changes occurred in the few lymphatics observed. Exudate cells were present in the lumen of these vessels; and, except for the presence of the coagulum and paucity of cells, they could be confused with blood capillaries.

### *Twenty-Four Hours*

Changes of little significance could be detected in the blood capillaries and lymphatic vessels of the inflamed tissue. Active passage of leucocytes through the capillary wall was still the rule. The connective tissue was so completely infiltrated by leucocytes that much of its architecture was obscured. A considerable increase in the number of lymphocytes was observed in capillaries as well as in the exudate. A majority of those in the exudate showed hypertrophy of the cytoplasm, vacuole formation and an eccentric position of the nucleus. A relative increase in the number of eosinophils was also apparent. Scattered examples of mononuclear leucocytes with pale-staining irregular nuclei were encountered in the vessels and exudate. Those in the exudate revealed an extensive hypertrophy of the cytoplasm and showed evidence of phagocytosis. The features of this latter cell type strongly suggested that they were metamorphosing monocytes. Striking changes were apparent in the net of fibroblasts and in the associated clasmacytes. Many of the fibroblasts were in some state of mitosis. The dividing cells were rounded, darkly staining and possessed but a few extremely delicate hyaline processes. Examples of daughter cells were found which gave no obvious physical continuity with the cells of the net. The large majority of clasmacytes were either free in the exudate among the leucocytes

or were associated with the net without apparent adherence to it. Unless great care was exercised, the dividing fibroblasts (rounded and darkly staining) could be easily confused with the clasmatoctyes. The cytoplasm of clasmatoctyes free in the exudate was abundant and filled with vacuoles of various sizes. The nucleus was dark, without texture and indented. Clasmatoctyes still associated with the net gave the appearance of being stretched and contained fewer cytoplasmic vacuoles. No mitosis was observed. Some of the vacuoles of these cells contained granular debris.

#### *Forty-Eight Hours*

Connective tissue spread preparations were difficult to make in this period. The tissue contained much fluid, but resisted all efforts to tease the tissue into thin films. Restricted but satisfactory areas for study were present.

Few changes could be observed in the capillary endothelium. The cells appeared less swollen than in the preceding periods and in general the lumen surface of the cells was less irregular. Large numbers of mononuclear leucocytes (chiefly lymphocytes and a much smaller number of monocytes) filled the lumen of the vessels and could be seen migrating to the exudate. Few heterophils were found in the vessels at this time and none were observed in migration. Lymphatics appeared unchanged except that they now contained many mononuclear cells and degenerating heterophils.

The lymphocytic and monocytic character of the emigrant mononuclear cells was of short duration. A striking and rapid hypertrophy involved these cells as quickly as they reached the exudate. Transitions from the characteristic cell type to amoeboid cells with abundant vacuolated cytoplasm and dark irregular nuclei occurred in a zone 15-20 micra from the capillaries. Everywhere throughout the exudate were evidences that these hypertrophied cells had become exceedingly active macrophages. The formerly abundant heterophils of the preceding periods were rapidly degenerating. Phagocytosis of their debris by the macrophages was of general occurrence throughout the tissue.

The formerly distinct fibroblast net now showed areas of disruption and disorganization. Unattached groups of from three to eight fibroblasts, as well as single rounded cells, were a common feature in the exudate. Fine cytoplasmic processes attached the adjacent surfaces of the cells of a group, but they were otherwise devoid of processes. The cytoplasm stained darker than is normal and was distinctly vacuolar. No cell division was observed in the liberated cells. Numerous mitoses were observed, however, in the intact net. Free cells were observed immediately surrounding these groups which showed distinct fibroblastic characteristics and appeared to be in all stages of activation leading to phagocytosis. The abundance of histogenous and hematogenous macrophages and their transitional forms made identification of a fibroblast-derived macrophage difficult, if not impossible, because of cytological limitations of the technique.

#### *Seventy-Two Hours*

Capillaries during this period appeared to be undergoing a mild degree of repair. A higher incidence of mitosis was noted in the endothelial cells. The

vessels still contained mononuclear leucocytes predominately; however, fewer of these cells were observed passing to the exudate. No changes of significance were observed in the lymphatics.

The cellular exudate consisted mainly of large numbers of macrophages filled with the debris of heterophils. Only scattered examples of non-phagocytized heterophils remained.

Restoration of the fibroblast net appeared to be in progress as evidenced by the large number of dividing cells. No free fibroblast groups, single cells, or transition forms could be identified in the exudate in this period.

#### OBSERVATIONS ON THE RÔLE OF THE FIBROBLAST IN INFLAMMATION AS EXHIBITED BY CONNECTIVE TISSUE SMEAR PREPARATIONS

The study of inflamed subcutaneous tissue of 1 to 12 hours duration was accomplished satisfactorily by use of sectioned material and connective tissue spread preparations. The cytological features of the tissue in general were followed with comparative ease and no particular problems were encountered in understanding the behavior of the fibroblasts for these periods. The highly cellular stages from 24 to 72 hours duration, however, presented considerable confusion and difficulty in the interpretation of reacting cells. The 24 to 48 hour period, in particular, exhibited changes in the fibroblast net which strongly indicated the liberation of individual cells and groups of cells to the exudate and their subsequent hypertrophy to macrophages. Significant cytological clues were lacking which would permit, with some degree of certainty, an orderly tracing of the process.

Gross examination of the tissue in the 24 to 72 hour period revealed a diffuse edema and numerous foamy masses. It was found that by pricking these foamy masses a sufficient amount of exudate could be collected on a thin section spatula to make a smear in much the same manner as a blood smear is made. The smears were stained with Wright's Blood Stain and studied. All of the cells in the exudate of the 24 to 48 hour period were in a state of fine preservation. The heterophils were the only cells subject to injury. Lymphocytes and their hypertrophied forms leading to macrophages could be followed unmistakably. On all the slides there were well-preserved fibroblasts; many of them in groups still attached to one another, and individual ones. The generally recognized cytological criteria, characteristic of blood smears and imprints of bone marrow, were applied to the study of exudate fibroblasts. Smears of the exudate for the periods of 1 to 12 hours duration did not contain fibroblasts. The ensuing description will concern the 24 to 72 hour periods when they were readily obtained in the exudate.

The fibroblast shown in figure 1 is representative of the appearance of most exudate fibroblasts of the 24 hour period. Occasional examples were found in which the cytoplasm contained numerous small vacuoles. Three or four blunt cytoplasmic processes were usually present on single cells, whereas the cells of a group exhibited a rounded cytoplasm and many delicate processes. The cytoplasm stains light blue and contains delicate flaky patches.

The nuclear membrane of the ovoid nucleus is barely perceptible. Some

cells contained a more nearly round nucleus than the one depicted in figure 1. Rounded nuclei were more commonly found in cells arranged in groups. The extremely fine chromatin granules are arranged in patches and form dark and light areas throughout the nucleus. In other similar cells observed the chromatin granules show a linear alignment which produces a reticulate texture of the nucleus. Regardless of the arrangement of the granules, the nucleus stains a light lilac color. The cell in figure 1 shows three light nucleoli; other cells were observed with as many as five.

A number of cells were observed in the exudate of the 48 hour period which were round except for one broad process. In each instance these cells exhibited a large oviform nucleus and slightly basophilic cytoplasm. Such a cell is shown in figure 2. The lilac-colored parachromatin, delicate nuclear membrane and nucleolus mark a similarity to the nucleus in figure 1. Likewise the cytoplasm, though altered in external form, sharply resembles that shown in figure 1. Examples of these cells were observed attached to a group of fibroblasts by the one remaining process. Recognition of these cumulative characters led to their identification as altered fibroblasts. Macrophages of hematogenous origin and their earlier transition stages were easily distinguished from the cell type shown in figure 2 by their abundant active cytoplasm and their coarse basic-staining nuclei. Clastmatocytes which were free in the exudate also showed an abundant vacuolated cytoplasm, but could be readily identified by their coarse nuclei which showed a mixture of lilac parachromatin and purple chromatin.

Figure 3 represents an altered fibroblast which has withdrawn all but one short process and is essentially a round cell. The cytoplasm on one side of the nucleus exhibits several small hyaloplasmic vacuoles. The nucleus is fibroblast in character (compare figures 1, 2 and 3). Many transitional stages leading from the condition shown in figure 2 to that of figure 3 were observed. The figures presented here were selected to show the trend of alteration involving these cells.

Completely rounded cells exhibiting a condensed oviform nucleus were common although not numerous in the exudate of the 48 hour period (figure 4). Recognition of transition stages from the condition shown in figure 3 to that of figure 4 required constant reference to the cytological features of the cell types shown in figures 1, 2 and 3 as well as the transition forms of the hematogenous macrophages. The latter were readily segregated by virtue of their indented, basic-staining, lymphocytic nuclei.

The cell in figure 4 shows a distinct nuclear membrane and a more compact chromatin arrangement. Three pale-staining nucleoli can be observed; these, however, were not constant features in all cells of this type. Although smaller and more darkly staining, the nucleus in general retains its fibroblastic character. The cytoplasm presents a mottled appearance and shows a somewhat deeper basophilia than in the cells previously described. Numerous small hyaloplasmic vacuoles and several large ones have made their appearance in the cytoplasm.

Attention has been directed to the progressive alterations of exudate fibroblast from characteristic stellate cells to contracted rounded ones. The cell in

figure 4, although fibroblastic in character, reveals a cytoplasm, which, by the usual signs, suggests preparation for phagocytosis. A number of round cells were observed approximating the condition shown in figure 4 except for an obvious diminution of basophilia in the cytoplasm. Many of these cells contained considerable debris of heterophils within their cytoplasmic vacuoles. A cell representing these features is shown in figure 5. Cells of this type were not numerous in the exudate and were relatively inconspicuous. They seemed capable of phagocytizing fine particles such as heterophil granules and particles of nuclear debris. Many of the cells contained so many heterophil granules that they could easily be mistaken for heterophil myelocytes. Although the nucleus appears contracted and the chromatin granules condensed, the texture is still delicate. These features of the nucleus, together with its staining quality, indicate the fibroblast derivation of the cell.

The exudate of 72 hours did not lend itself well to the making of smears. The large number of macrophages in the exudate at this time showed a tendency to rupture or overlap. A few cells representative of figures 3, 4 and 5 were observed, but debris of ruptured macrophages in the preparations would not permit their study.

#### DISCUSSION

Of all the cell types present in the loose connective tissues of the body, no cell has been quite as obstinate as the fibroblast in refusing to display clearly its potentialities under different physiological conditions. Today there is little agreement concerning the rôle of the fibroblasts in the inflammatory reaction. Moreover, the fibroblast has exhibited a particular elusiveness in displaying its relation to other cells of its kind. There is little agreement as to whether there is cytoplasmic continuity from one fibroblast to another (syncitial arrangement) or whether each fibroblast is a distinct cellular entity capable of independent expression. The solution of these problems would not only further clarify the abilities of the fibroblasts as a cell type, but would also lead to a more complete understanding of the cellular sequence involved in the inflammatory process. The following discussion of literature pertinent to these problems as well as observations in the present investigation will provide, it is hoped, a basis for appreciating the need for continued investigation of the subject as a whole.

Observations on the fibroblast net throughout the early periods (1 to 12 hours) of the present investigation revealed few noteworthy changes, with the exception of a progressive increase in the number of mitoses. These features of the early periods coincide with the views of Maximow (1905) who regarded the fibroblasts as a terminal differentiation of mesenchyme and of limited potentialities. According to him, the definitive nature of the fibroblasts expresses itself in inflammation by their inconspicuous rôle of reproduction and growth in number without undergoing morphological change. Alfejew (1924) and Tschachin (1913) agree with Maximow regarding the origin and stable nature of the fibroblasts.

In the 24 hour period the number of mitoses in the net showed an increase

over the earlier periods. A considerable number of the dividing cells resulted in the liberation of daughter cells to the exudate without obvious depletion of the fibroblast net. Many of these liberated cells appeared in smears of the exudate as shown in figure 1.

Stimulation of the fibroblast net appeared profound in the 48 hour period. A large number of mitoses occurred coincident with the liberation of groups of fibroblasts and individual ones from the net. Foci of disorganization and disruption could be identified within the net at such points where cells were being liberated. These events were easily discernible in both spread and sectioned preparations. These preparations also revealed alterations in the liberated fibroblasts. Further amplification of the events occurring were obtained from the connective tissue smear preparations which exhibited a series of transitions involving the liberated cells. Progressive transitions from stellate fibroblasts to rounded active phagocytes were observed (figures 1 to 5).

The features of the 48 hour period relative to the fibroblasts are not in agreement with the views of Maximow (1934). He did not consider fibroblasts capable of phagocyte formation. According to him, the large number of phagocytes which engage in the inflammatory reaction are derived in the main from lymphocytes and monocytes which hypertrophy following their migration from the vessels. He believed that clasmacytocytes constitute the sole remaining source of phagocytes. The 24 and 48 hour period in the present study revealed clearly that the vast number of phagocytes in the exudate were derived from hypertrophied monocytes, lymphocytes and clasmacytocytes. However the liberation of fibroblasts to the exudate and their subsequent metamorphosis to phagocytes was a striking feature of the 48 hour period.

Von Möllendorff and Von Möllendorff (1926) regarded the fibroblasts as a tissue reservoir from which large numbers of phagocytes arise. From observations on connective tissue spread preparations of animals injected with trypan blue, they concluded that in inflammation the fibroblasts are the chief reacting cells and that they respond as a system. The stimulated form of the fibroblast gives rise, according to them, to histiocytes, macrophages, polymorphonuclear leucocytes or more fibroblasts. The degree of stimulation dictates the cell type which will form. They further observed that stimulated fibroblasts divided by amitosis. Knake (1927) investigating the connective tissue reactions of rabbits injected with india ink also observed the formation of phagocytes and granular leucocytes from stimulated fibroblasts. She also noted that amitotic division led to a depletion of the fibroblasts of the region and an increase in histiocytes by direct transformation.

Through the use of spread and sectioned preparations Lindsey (1929) studied the reactions of subcutaneous tissue to experimental tuberculosis in the guinea pig. She regarded the fibroblasts as primitive cells closely related to primitive mesenchyme on the basis of their formative powers. She was able to trace a closely graded series of transitions in the transformation of fibroblasts into clasmacytocytes, polyblasts, monocytes, epithelioid cells, plasma cells and small histiocytes resembling lymphocytes. She observed mitosis as the prevailing type of cell division.

Although fibroblasts were observed to transform into phagocytes in the 48 hour period and provide cells to the exudate, the abundant developmental potentialities which Von Möllendorff and Von Möllendorff, Knake and Lindsey ascribe to stimulated fibroblasts was not observed. Spread and sectioned preparations revealed many cell transitions of a doubtful nature as to their trend. Utilization of the smear preparations made it possible in most cases to identify distinct and constant cytological features by which transitional forms of the reacting cells could be distinguished. No cells were observed to be liberated from the fibroblast net during the 72 hour period. Some examples of transitions of fibroblasts were observed in the exudate, but in general the smear method was not practical for this period. Mitosis appeared to be restricted to repair of the fibroblast net.

Maximow (1926) postulated the existence throughout the connective tissue of undifferentiated mesenchyme cells which were morphologically indistinguishable from fibroblasts. To these cells he attributed full developmental potentialities. He described these cells as being most numerous in the vicinity of blood vessels. In the present study numerous foci in the net were observed where cells were being liberated. No difference could be detected in activities of the fibroblasts in the proximity of a vessel or some distance from it. The presence of cells morphologically identical to fibroblasts but different in respect to developmental potentialities may well be present in the connective tissues of the body. However, since there are no adequate cytological features by which fibroblasts and undifferentiated mesenchyme cells may be distinguished from one another, it would appear more satisfactory to fuse their identity into a single morphological type (fibroblast), and at the same time allow a greater range of developmental potentialities to the morphological type.

#### CONCLUSIONS

1. The fibroblasts of loose connective tissue can be stimulated to produce phagocytic exudate cells.
2. The number of fibroblast-derived phagocytes in inflammation appears to be small as compared to the large number of macrophages derived from blood lymphocytes, monocytes and tissue clasmacytocytes.
3. There is little evidence that fibroblasts are able to produce granular leucocytes or any other cell types in inflammation other than phagocytes.
4. The period of greatest fibroblast activity in phagocyte production appears to coincide with the period in which large numbers of mononuclear leucocytes leave the vessels and hypertrophy to phagocytes.
5. Connective tissue smear preparations are a helpful adjunct to sectioned and spread preparations in following cellular transitions during the highly cellular stages of inflammation.

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#### EXPLANATION OF PLATE 8

Fig. 1. A fibroblast from a connective tissue smear preparation of the 24 hour period. Note the delicate texture of its nucleus and the presence of several faintly-staining nucleoli. The cytoplasm retains a stellate appearance.  $\times 2000$ .

Fig. 2. A fibroblast from a smear preparation of the 48 hour period. This cell has withdrawn all but one of its processes. The delicate chromatin granules of the nucleus show areas where they are in linear alignment.  $\times 1500$ .

Fig. 3. A fibroblast from a smear preparation of the 48 hour period. This cell is round except for a short process. The nucleus is delicate and no obvious nucleoli can be seen. Several small hyaloplasmic vacuoles can be seen in the cytoplasm at one side of the nucleus. This type of cell could easily be confused with a lymphocyte or monocyte in spread and sectioned preparations.  $\times 1500$ .

Fig. 4. An altered fibroblast from a 48 hour smear preparation. No processes are visible and the vacuolated appearance of the cytoplasm suggests that this cell is preparing for phagocytosis. The nucleus is oviform in shape and possesses several nucleoli.  $\times 1500$ .

Fig. 5. An example of a fibroblast-derived phagocyte from a smear preparation of the 48 hour period. Phagocytosis is in evidence by the heterophil granules which this cell has taken into its cytoplasm. Although the nucleus is condensed, it still retains its fibroblast characteristics. Cells of this type, while not numerous, were common in the exudate of the 48 hour period.  $\times 1500$ .

PLATE 8



2



3



4



5



# THE VENEREAL DISEASE PROBLEM IN THE UNITED STATES IN WORLD WAR II<sup>1</sup>

BY WILLIAM L. FLEMING

*School of Public Health, University of North Carolina*

## INTRODUCTION

As the general public has only recently become aware, the venereal diseases constitute one of our most important health problems in peace times. This problem has always in the past been aggravated by war both in the army and in the civilian population.

Statistics in the Office of the Surgeon General on the annual venereal disease attack rate in the U. S. Army (1) show increases with all our previous wars, the highest peak being reached with a rate of 215 per thousand men immediately after the Civil War. The increase with World War I, however, was hardly a significant one: due to the excellent control efforts, the rate for 1917 (107 per thousand) was not much above rates for the preceding four years and in the post war period the rate dropped precipitously instead of rising.

A very real problem which still confronts us during the present war is whether we can adequately control the venereal diseases. Our knowledge and facilities are greater than they ever have been, but we have to fight all of the adverse effects on the venereal disease problem of a long war fought in foreign countries.

## EXTENT OF THE PROBLEM

The importance of the venereal diseases should be well enough understood by now so that it should not be necessary to take the space to state reasons. Suffice it to say that syphilis has been very aptly called "the great destroyer of life" and gonorrhea "the great preventer of life." The extent of the problem will be discussed briefly.

*Syphilis.* Due to statistics obtained from the examination of selectees and volunteers during this war, we have a more accurate idea of the prevalence of syphilis than we have ever had before. Vonderlehr and Usilton of the United States Public Health Service report (2) that among the whites and negroes 21-35 years of age in the first 2,000,000 draft board examinations 45.3 per thousand (4.5%) had syphilis; they estimated the prevalence rate of syphilis in the entire male population of the United States between 21 and 35 to be 47.7 per thousand (4.7%). The rate of prevalence for negro selectees (21-35 years) was 252.3 per thousand (25.2%), for white selectees (same age group) 17.4 per thousand (1.7%). Expanding the draft figures to the general population of the country (all races and age groups) Parran and Vonderlehr in 1941 (3) estimated that 2.4% or 1 in 42 have syphilis at the present time. In North Carolina corrected rates for the male population 21-35 based on the selectee serologic tests for syphilis were,

<sup>1</sup> Based in part on a paper delivered before the North Carolina Academy of Science, April 30, 1943.

according to Vonderlehr and Usilton (2) 2.8% for whites, 23.8% for negroes, and 8.5% for both. Another source of information on the prevalence of syphilis is the number of cases under treatment; Heller (4) reports that in 1943 "almost" 600,000 cases were reported (new and old cases).

*Gonorrhea.* We have no reliable estimates of the prevalence of gonorrhea. Estimates of the prevalence of gonorrhea in the general population based on data derived from draft examinations have indicated a prevalence rate of considerably less than 1% (5). The discovered prevalence rate among draftees was undoubtedly too low due to inadequate methods of examination. It gives no real idea of the gonorrhea problem, nor would the true prevalence rate since gonorrhea tends to be a self-limited disease, particularly in the male. The attack rate (number of new cases occurring annually) of gonorrhea has been estimated to be 4-5 times that of syphilis in the civilian population and in army camps it is not infrequently 7-10 times greater.

*Other Venereal Diseases.* We have no reliable figures for the incidence or prevalence of chancroid, lymphogranuloma venereum, or granuloma inguinale in the civilian population.

#### PRIOR TO WORLD WAR II

There was very little organized venereal disease control in this country prior to World War I. Use of potent antisyphilitic drugs by private physicians was not too common and public facilities were rare.

During World War I an energetic venereal disease control program both in the army and in civilian communities (mainly around army posts) was instituted. Considering the situation, a remarkably good job was done. The U. S. Army had less venereal disease than any other army in the war. In spite of this record the U. S. Army quoting McNutt (6) "lost seven million days to venereal disease — 338,746 officers and men, the equivalent of 23 divisions under treatment for syphilis and gonorrhea."

The venereal disease control program had been identified as a war problem to such an extent that by 1923 federal funds were withdrawn and the control program soon became very poor in most of the country.

From 1923 to 1936 there was a gradual rekindling of interest in the venereal diseases but little was accomplished beyond improvement in the methods of treating syphilis. At this time, largely due to the efforts of Surgeon General Parran of the United States Public Health Service, interest was aroused in the problem. This interest soon led to the stimulus of federal funds in the control program which was well on its way by the outbreak of this war. The number of venereal disease clinics in the country increased from 713 in 1936 to 3,088 in 1942 (7). The volume of the number of treatments administered annually increased from 2,122,000 in 1936 to 10,178,000 in 1940 (7).

North Carolina was fortunate in that it was able to get off to an early start in its venereal disease control program. Before federal funds became available in any quantity (1938), the State Board of Health had begun to receive an annual grant of funds for venereal disease control from the Smith Reynolds Foundation.

Starting in 1936 with a small number of inadequate clinics in the larger cities, the number of clinics in the state had increased to 311 in 1943, or about one-tenth of the number of clinics in the nation.

The trend of the venereal disease problem prior to the war was difficult to judge because of the difficulty of obtaining reliable figures on the prevalence or incidence of these diseases. There seems little doubt, however, but that syphilis had declined in prevalence before the war. Parran (8) reports that in 1917 "clinical syphilis (serologic tests were not done) caused the rejection of approximately 10,000 men per million examined." In the first million men examined in the current draft only 1,482 men were reported to have clinical syphilis, less than  $\frac{1}{7}$  the figure for World War I. There are various reasons for doubting that syphilis has declined as much as this figure would indicate but there is little doubt that this great discrepancy indicates a decrease.

We do not have reliable data on the trend of gonorrhea, the other most important venereal disease. It is to be doubted that it had decreased significantly since effective treatment applicable to clinic use (sulfonamides) had not been in general use very long before the outbreak of war.

#### WORLD WAR II

Most of the factors associated with war which tend toward an increase in the prevalence of venereal diseases are due to the coincident tremendous social upheaval and have been present to an unusual extent in this war. Masses of people have been shifted from one location to another to meet the demands of war industry with the consequent disruption of family ties and community life. More people have more money to spend. There is a general tendency to a let-down in morals due to the general upheaval and to war psychology. Millions of young men have been taken out of their accustomed environments into the Armed Forces with the abnormal environment of camp life, with all too frequent inadequate recreational facilities, and battle conditions in foreign countries. The underworld with its prostitution and allied rackets has done its best to muscle into military camp and war industry areas.

Many changes have been made to meet the situation. In September 1939, shortly after the outbreak of war in Europe, and in anticipation of unusual conditions in this country, representatives of the Army, Navy, and United States Public Health Service met and drew up what has been called the "eight point agreement." This agreement defined the responsibilities of military and health organizations in the venereal disease control program and set up a scheme of co-ordination of effort. This plan was ratified by the State and Territorial Health Officers in May 1940.

In July 1941 the "May Act" was passed by Congress. This act permitted the declaration of prostitution as a federal offense within the vicinity of military establishments when local control had failed. It has been put in force in only two areas: in North Carolina in the Fort Bragg area and in Tennessee in the Camp Forrest area.

In general, good progress has been made in the wartime program. Some

initial friction between the agencies involved in civilian venereal disease control with those of the Armed Forces was soon overcome. The advent of the use of specialized personnel in this field in the Army and Navy has made for considerable progress. Prostitution, which had gotten off to a roaring start in the vicinity of many military camps and war defense areas, has been fairly effectively dealt with in most places.

Great changes have occurred in treatment facilities and in methods of treatment of both syphilis and gonorrhea. The increase in population shifts made even more unsatisfactory the protracted standard weekly treatment of syphilis under which it has been estimated (4) that only 25% of patients with infectious syphilis receive sufficient therapy. This situation has been met at least in part by the development of much more rapid methods of antisyphilitic treatment using the same drugs—arsenicals and bismuth as used in the standard treatment scheme. These methods of treatment require hospitalization or very close supervision, so "Rapid Treatment Centers" (hospitals) for syphilis and other venereal diseases have been set up under the auspices of the United States Public Health Service with Lanham Act funds. Forty-six of these centers were in operation by April 1944 (9). Infected transient girls and prostitutes can be handled particularly well in these hospitals.

Treatment of gonorrhea with sulfonamides which had been introduced shortly before the war has been greatly expanded. Just when this form of therapy seemed to be losing some of its first effectiveness, penicillin was found to be very effective in the treatment of gonorrhea and has been widely used in the Rapid Treatment Centers. Penicillin has also been found to be effective against syphilis and is being tried out on a large scale in hospitals and rapid treatment centers; its lack of toxicity as compared to the arsenicals used in antisyphilitic therapy is very encouraging.

Another development which has resulted in a large saving of man-days lost from venereal disease in the Armed Forces has been the practice of cutting hospitalization to a minimum and administering as much of the treatment as possible on a "duty status."

The results of the venereal disease control program have been encouraging. Rates in the Armed Forces have risen only slightly or have been reduced. Turner and Brumfield (10) point out that the total venereal disease rate in the United States Army was 29.6 per thousand in 1939, the lowest recorded up to that time; 42 in 1940; and in 1941 and 1942, at or slightly below that level. Even with this increase the rate was less than half of World War I rates. Turner and Brumfield (10) further point out that when venereal diseases acquired prior to induction are eliminated, the rate is in all probability lower than the lowest peace time rate. In addition, by treatment on duty status man-days lost from venereal disease have been reduced so that Turner and Sternberg (11) report that "days lost per thousand men annually dropped from 1278 in 1940 to a level of approximately 400 at the present time" (October 1943). The Navy has reported (12) a substantial lowering of the venereal disease rates for 1942: 36 per thousand. This figure represents a decrease of 29% from 1941 and of 55% from 1940.

However, too many soldiers and sailors are still being infected, too many infections are still being discovered in selectees, too many defense workers are losing time because of venereal diseases. The venereal diseases continue to rank high as a cause of man-days lost from sickness in the Armed Forces and in the civilian population. We have no right to feel that we have accomplished any more relatively, if as much, as was accomplished in World War I.

#### PROBLEMS

Many problems still face us. Some of them are: 1) The Army has discovered that the lowering of its venereal disease rate by the campaign against prostitution has been partly offset by an increase in infections due to pick-ups, waitresses, tavern and dance hall girls, and the like, who at the most are only part-time prostitutes and many of whom are teen-age girls.

2) The Army is now encountering a different and more difficult problem in controlling venereal disease among the troops on the continent of Europe. Can our present fairly low rates in the military forces be maintained or better lowered? Also will the situation get out of hand in the civilian population due to shifting of population, shortage of public health and medical personnel, etc.? Our danger in this regard is brought forcefully home to us by the experience of England. In that country where more had been accomplished in syphilis control before the war than in ours, the long downward trend in the number of syphilis infections has been reversed and almost 50% more cases were reported in 1941 than in 1939. It has been estimated that if the number of service infections were added, the increase would be 70% (13). Also in Sweden, which before World War II had led the world in controlling syphilis, the downward trend of many years has been reversed and in 1943 more than three times as many new syphilis infections as in 1937 were reported (14). We do not have the evidence in this country that venereal diseases have actually increased in the civilian population, but there is a real danger that they may in spite of our increased efforts at control.

3) Finally, the problem remains of what will happen to our venereal disease control program after the war. Will we repeat even in part our mistake of World War I when as a result of identifying the program too much as just a war effort, it was dropped almost completely after the armistice?

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# IMMUNITY RELATIONS IN HUMAN CESTODE INFECTIONS

BY JOHN E. LARSH, JR.

*School of Public Health, University of North Carolina*

## INTRODUCTION

Most of the important parasites of man and domestic animals have been studied from an immunological point of view. Studies during the first 30 years of this century were concerned chiefly with practical diagnosis of important infections, but in addition our knowledge of other aspects of the subject was improved likewise. It was pointed out, for example, that at least partial immunity was acquired following recovery from various parasitic infections and that special specific substances are present in both normal and immune sera. During the past 15 years, and particularly the past decade, practical and theoretical problems of immunity to parasitic infections have been attacked with great interest, as is exemplified by the voluminous literature. In a large part these studies have centered on protozoan and helminthic diseases and of the latter the nematodes (roundworms) and cestodes (tapeworms) have been given most attention.

It is the aim of the present paper to consider briefly some of the important points in the study of immunity to cestodes. Although the first extensive experimental investigations demonstrating immunity relations for a helminth were carried out with a rat-cat parasite (Miller, 1930 and later), and a great deal of later work was done on non-human forms, space does not permit consideration of these interesting studies. Therefore, discussion will be limited to human cestode infections which show the clearest evidence of immunity, viz., *Taenia* cestodes, *Echinococcus*, and *Hymenolepis*. Again space allows mention of only a few representative papers which illustrate most clearly the various topics considered.

## GENERAL ASPECTS OF IMMUNITY

The term immunity as used here refers to resistance or lack of susceptibility of hosts to infection with cestodes. This immunity may be due to a variety of factors not understood well, such as physical and chemical conditions in the body, suitable food, physiological processes of the host, and particular defensive mechanisms of the animal either present naturally or acquired following infection. *Types of immunity:* (1) *Natural resistance.* This type of resistance is present in the body at birth and is the result of inherited morphological and physiological properties. In other words, an individual possesses a degree of natural resistance against certain infections merely as a result of belonging to a given species and race. Only certain species of hosts are susceptible to a particular cestode parasite, and the number of different species a parasite infects normally varies widely. For example, some cestodes, such as the broad tapeworm (*Diphyllobothrium latum*) which has been found in the adult stage in man, dogs, cats, bears, foxes, etc., can adjust to many animals. This is an example of loose

host-parasite specificity. Other cestodes are strict in their host relations and infect a single species, as is illustrated by the beef tapeworm (*Taenia saginata*) which occurs as an adult only in man. Hosts which are suitable for the establishment and maintenance of a parasite are called normal and those which have a natural immunity to a given parasite are termed abnormal. Many abnormal hosts display an absolute immunity but in other cases the immunity may be only partial as shown by: (1) completion of part of the life cycle without attainment of sexual maturity, (2) shorter life span than in the normal host, (3) lower rates of infection, etc. In final analysis, little is known of the mechanism of natural resistance but it is obvious for a parasite to infect a given host it must be equipped to contact that host and upon entry meet with an adequate physiological environment.

(2) *Age resistance.* When older animals, although not infected previously, show a well defined resistance against parasites to which they are susceptible when immature, it is usually referred to, for lack of a better name, as "age resistance." Several theories have been advanced in way of explanation for this peculiar resistance, but in considering all cases a variety of factors, many yet unknown, are involved. Since the host is thought to possess this resistance without reference to prior association with the parasite, it is considered a form of natural resistance.

(3) *Acquired immunity.* This type of immunity may be of two kinds, active and passive. If the animal is infected with (natural), or vaccinated against (artificial), a parasite and in response elaborates specific protective substances (antibodies) the resulting resistance is termed active. The cells of the animal are stimulated by the foreign material (antigen) and in way of protection actively forms neutralizing bodies. On the other hand, if these antibodies are produced in one animal, the donor, and transferred to another, the recipient, the resulting resistance against the homologous parasite is termed passive, since the cells of the recipient were not aroused in the process. The antibodies introduced are foreign to the recipient and as such are eliminated soon, which accounts for the passive, ephemeral, nature of this type of resistance. The result is the same whether the transfer is natural (mother to offspring) or artificial (blood or serum from one host to another).

Hosts with acquired resistance to a parasite may, in some cases, prevent all development, or, more often, cause a substantial reduction in the number of worms that develop, prevent normal growth, and shorten the life of the infection.

The mechanism of acquired resistance to cestodes is concerned mainly with the larval stages in tissues, since the adults in the intestine have been shown repeatedly not to elicit immunological responses to an effective degree. In many instances the immune responses to the tissue invaders have been shown to operate in much the same manner as against bacterial and other infections. Because of their large size, these forms often destroy tissue in a mechanical way by penetration, but in addition metabolic products are given off which irritate the cells. These irritating substances (antigens) stimulate the cells to produce neutralizing bodies (antibodies) which, in many cases, are of great value in arresting the

infection. Once arrested by these substances, the parasite is more easily attacked by the cells which tend to destroy, encapsulate, and, in time, even to absorb them. This cellular response in non-immune hosts is too feeble generally to arrest penetration and prevent establishment of parasites.

Acquired immunity can be induced naturally by cestodes during the normal course of their life cycles, especially by those stages that live or migrate in tissues or the circulatory system. In addition the cellular response of a host can be aroused artificially by injections of dead cestodes or their products. Whatever may be the source of the cellular stimulation specific antibodies are produced in an attempt to neutralize the antigen. Besides the local concentration of antibodies in the region attacked by cestodes, these protective substances circulate freely throughout the body in blood and lymph. This fact makes it possible to diagnose certain cestode infections by immunological methods, i.e., by *in vitro* means or by skin testing. In the former case a sample of serum containing the specific antibodies is mixed in a conventional manner with antigen prepared from the homologous, or in some cases a closely related, cestode. When antibodies come in contact with an antigen which caused their formation in the body certain specific immunological reactions take place. For example, in case of the ring-precipitin test which is used widely a small amount of the patient's serum is placed in a series of small tubes and a similar amount of a clear solution of antigen containing the cestode proteins is superimposed. If the serum contains specific antibodies a visible precipitate is formed at the juncture of the serum and antigen, whereas in control tubes with normal serum this white ring is absent. The complement fixation test is another widely used *in vitro* means of detecting antibodies against cestodes. In the case of the intradermal or skin test the antigen is injected into the skin of the patient to determine the presence in the circulation of antibodies. If these are present there is a reaction with the formation of a wheal or bleb which is not present when the antigen is injected into a person void of the cestode antibodies. The mechanism involved in these tests, whether they are performed *in vitro* or *in vivo*, is similar and demonstrates the presence of certain antibodies. In actual practice it is impractical to diagnose a cestode infection by these methods when eggs or portions of the worms can be recovered by simple fecal examinations. However, in the case of true tissue cestodes, e.g., *Echinococcus granulosus* and others in man, these tests have been of greatest value in diagnosis as is pointed out below.

#### IMMUNITY TO *TAENIA* CESTODES

*Taenia solium* and *Taenia saginata* are very large cestodes which occur in the adult stage only in man and have very similar life cycles. Segments of the worm escape in the stool and the infectious eggs are thereby made accessible to grazing animals. When ingested by the appropriate intermediate host a small embryo inside the egg escapes from the shell and after making its way by penetration into the circulatory system is deposited in connective tissue and fat around muscles and develops into a bladder-like larval stage. Man is infected with *T. solium* by eating raw or insufficiently cooked pork containing mature larvae

and in the same way he acquires an infection with *T. saginata* by eating beef. Other animals exposed to these larvae must possess a strong natural immunity but the mechanism involved is not known.

It has been noted frequently that the development of a single specimen of either of these cestodes in man appears to prevent the development of others of the same species. This condition lasts only as long as the infection. The reasons for this type of resistance are vague but it must be accompanied by some physiological change which is unfavorable for the establishment of other worms. There is evidence for this view in the numerous cases which show only a single worm although the ingestion of additional cysticerci (larvae) was known to be frequent. The intestine can accomodate more than one worm as shown by multiple infections on record so that mere crowding does not seem to be an all important factor. As would be expected by the fact that these parasites are lumen dwellers, there is no evidence that antibody production plays any part in this resistance. In contrast, the larval stages in the tissues produce strong immunity with a true antibody basis.

The larval stage of *T. solium* (*Cysticercus cellulosae*) occurs normally in hogs but can, and, in some regions, often does develop in man. In man these larvae have been found practically in every organ and tissue of the body but occur most often in subcutaneous layers and the brain. In the latter they can produce cerebral cysticercosis. In these locations the larvae are walled off by a tough capsule and their detection depends on roentgenograms or immunological methods. Of the latter, complement fixation and skin tests have been used successfully in a few studies. However, the best results in diagnosing the larvae of *T. solium* in both man and swine have been obtained with the precipitin test. Rothfeld (1935) used this method in diagnosing four cases of human cerebral cysticercosis. In two of these the diagnosis was confirmed later by excising larvae from muscles just under the skin and by sectioning brain tissue taken at autopsy. According to Culbertson (1941), Trawinski (1936) also used this test experimentally to diagnose infection in pigs.

The larval stage of *T. saginata* (*Cysticercus bovis*) occurs normally in cattle but has been seen very rarely in man (Faust, 1939). The immunity relations of these larvae in cattle have been studied in detail by Penfold and his co-workers in Australia (1936). A large number of animals was infected in a series of experiments and in some cases as many as 30,000 larvae scattered throughout the body developed in a single animal. They were able to demonstrate that acquired immunity results from infection. Twenty calves were allowed to graze on a sewage farm where infection had been produced frequently. Later these animals were immune completely to infections of 400,000 eggs each, and there was evidence that as few as five larvae would produce this immunity. In another study, seven calves were fed 400,000 eggs and between one and two years later were given a second infection. Autopsy findings showed that all the larvae were dead which had developed from the initial infection and there was no evidence whatever of reinfection, i.e., complete immunity had been acquired. The experiment was controlled by also feeding the second test dose to non-

immune animals. The authors suggest that natural immunization of calves would constitute an important control measure in regions like Syria, etc., where *T. saginata* is so common. Besides preventing human infection such a control would be an economic measure, since it would save a great loss of meat now discarded at inspection.

There is no evidence that *Taenia* adults stimulate antibody production, but the immunity to the larvae of both *T. solium* and *T. saginata* certainly has an antibody basis as the selected work above points out. In all probability a strong immunity to reinfection occurs in swine infected with *Cysticercus cellulosae*, just as in cattle infected with *Cysticercus bovis*, but no experimental data were found in support of this view.

#### IMMUNITY TO ECHINOCOCCUS

*Echinococcus granulosus* is a minute taeniod cestode found as an adult worm in the dog, wolf, jackal, and cat. The digestive juices possibly play a rôle in natural resistance, since the hydatid membranes are digested by many resistant animals but not by the susceptible dog or cat (Berberian, 1936). Eggs escape in the stool and when ingested by any one of a large number of possible intermediate hosts (sheep-optimum host-, cattle, pigs, etc., and man) embryos are liberated and carried by way of the circulatory system to various parts of the body (liver, lung, etc.) where they grow slowly into hydatid cysts or larval forms. These cysts contain many, sometimes thousands of tiny scolices or heads of the adult worm which are produced asexually. When tissue containing the mature cyst is eaten by the definitive (final) host each of the tiny scolices can attach to the intestinal wall and grow by a budding process to form a mature worm. In other words, each scolex is a potential tapeworm.

Studies by Turner, Berberian, and Dennis (1936) demonstrated production of a partial protective reaction against infection with the adult stage of *E. granulosus* in dogs injected with antigen prepared from hydatid cyst material. The animals used were street dogs which before use were shown by fecal examination to be free of *E. granulosus* infection. They were fed from 3000 to 5000 scolices removed from larval cysts. All but one of the non-immunized controls was infected and about 70 per cent of this group showed heavy infections with thousands of adult worms in the intestine. On the other hand, only about one-half of the animals injected with the antigen preparations could be infected and of those infected only 22-33 per cent showed large numbers of worms. As would be expected, the dogs receiving a larger number of antigen injections developed a higher degree of resistance. Because some of the immunized animals became heavily infected and some controls very lightly infected, most workers interpret these results to indicate the development of, at best, only a partial immunity. Again to demonstrate a clearer immunological picture one must consider the larval stage in tissue.

Inasmuch as hydatid cysts are scattered throughout the body, develop slowly, and are long-lived, it would be almost impossible to carry out experiments on superimposed infections since they could not be distinguished from initial infec-

tions. However, Turner, Dennis, and Berberian (1937) have conducted experiments in attempts to produce artificial immunity to these larvae in sheep. Dried scolices and germinating membranes of hydatids from sheep and cattle were prepared as antigen which just prior to use was suspended in a weak solution of phenol-saline. Serial injections of this material were made in the thigh muscles of experimental animals, some receiving different numbers of doses. A total of 115 animals known not to have been infected previously were used in the study and those given antigen showed several clear-cut evidences of a protective reaction resulting from the immunization.

In general, the total number of cysts counted in the immunized animals was far below that in the non-immunized controls. In animals killed at the same time after infection, the diameter of cysts in the immunized animals was 50 to 100 per cent less than in controls, the cysts were firmer and more calcified in the former, and the location of cysts in the liver differed, being most often superficial in immunized animals and deep in controls. In addition, there were many microscopical differences in cysts taken from the two groups. In all experiments, the immunized animals were able by a rapid and efficient "walling off" process to defend themselves against the growth of the parasites. The greatly thickened capsule resulting from this process and an early calcification of the cyst wall produced an efficient barrier leading to the death of the cysts. Undoubtedly this accelerated reaction in the immunized animals resulted from the production of antibodies. These protective bodies were absent in non-immune animals which were unsuccessful in preventing growth or in killing the cysts.

The presence of specific antibodies in hosts infected with larval stages of *E. granulosus* has been demonstrated repeatedly by immunological tests. These tests were the first applied to any helminthic infection and have proved to be of great value in diagnosis. Two tests, complement fixation and intradermal, have been studied and used most extensively. The complement fixation test in the hands of many different workers has proved to yield favorable results. Fairley (1922) using fresh hydatid fluid obtained from active cysts that showed no signs of degeneration devised a very successful method. He obtained positive results in 84 per cent of 83 patients infected with hydatid and uniformly negative results in 917 persons, some of whom had syphilis or helminthic infections but not *Echinococcus*. Others have obtained as high or higher percentages of positive reactions. However, the specificity of the reaction has been disputed by several investigators. Rose and Culbertson (1939 and 1940) summarized evidence which indicates that the test is not species but group specific, meaning that positive reactions result when the antigen used is prepared not only from the same species but from any one of many related species. They suggest that antigen prepared from larval stages of *Taenia pisiformis* in rabbits or *T. taenia-formis* in rats can be substituted when hydatids are difficult to procure. Success with this test depends largely on the skill of the technician but when proper methods are followed it gives a high percentage of accuracy (Culbertson, 1941).

The intradermal test which now bears his name was used first by Casoni (1911-12) for the diagnosis of Echinococcosis (Culbertson, *ibid.*). A delayed

reaction was noted in 3 to 12 hours after injecting a carbolized cyst fluid. Since then a great deal of work has been done and this test, which may give either an immediate or delayed reaction, and which yields a high percentage of correct positive reactions, is considered the most valuable single procedure for the diagnosis of hydatid disease in man. The positive reaction is related to a hypersensitivity to cyst fluid produced by the presence of the cyst. The reaction has been found not to be specific, since it is positive for any species of *Taenia*, including the large human forms. That is, antigens suitable for eliciting reactions in cases of Echinococcosis can be derived from many cestodes, including in addition to *Echinococcus*, *Taenia serrata*, *T. saginata*, *T. taeniaformis*, etc. (Culbertson and Rose, 1941). Of course, intestinal infections can be ruled out by other simple tests and thus the value of the Casoni test is not decreased.

#### IMMUNITY TO HYMENOLEPIS

The dwarf tapeworm, *Hymenolepis nana*, which occurs in gerbilles, certain monkeys, and man is regarded by many as the most common human cestode. A form indistinguishable morphologically, and often designated as *H. nana* var. *fraterna*, has a wide distribution in rats and mice. Shorb (1933) found differences in the strains from these rodent hosts which strongly suggest they are physiological varieties. The same suggestion concerning the human form has been advanced but, as yet, experimental proof is lacking. There is also some suggestion that different strains occur within the same host (Larsh, 1943c).

The life cycle is peculiar because of the ability of this cestode to infect directly without development in an intermediate host. When infectious eggs are ingested by a susceptible host, the embryos escape and penetrate actively into intestinal villi where, in about four days, they develop into mature larval forms called cysticercoids. These break out of the intestinal tissue into the lumen and are carried farther down where they attach and grow into adults in two to three weeks. When fully mature, end segments are given off which during passage break up in the bowel liberating thousands of eggs. These occur in the stool and require very little, if any, incubation before becoming infectious. That this parasite can also develop in intermediate hosts (fleas, beetles) was demonstrated by Bacigalupo (1931). Direct development in a single host, the only method as yet demonstrated in this country, appears to be a recently acquired evolutionary change.

Studies have been made in rodent hosts of certain factors, such as age, diet, concurrent infection, etc., which influence the natural resistance to this cestode. Age resistance is certainly of some significance. Shorb (ibid.), Hunninen (1935b), and Larsh (1944b), have shown that mice more than five months old are much less susceptible than those of two months. To show the difference in susceptibility, Shorb (ibid.) made counts of adult worms in the two groups following similar infecting egg doses, but a more accurate method of counting mature cysticercoids in the villi was devised by Hunninen (1935a) and, unless otherwise specified, this was the experimental procedure used in studies carried out after the method was introduced. It also has been observed that persons

of the five to fourteen year age group are infected most frequently (Otto, 1936). The mechanism involved in this resistance is unknown, but if young mice are splenectomized and not infected until they are more than five months old the ability to resist infection is interfered with to a point where many are as susceptible as young controls (Larsh, *ibid.*). Associated with this susceptibility is a well pronounced but unexplained anemia which undoubtedly has something to do with the condition. Besides normal resistance associated with old age in mice, it has been noted by several workers (Shorb, *ibid.*, Hunninen, 1935b, Larsh, 1943a) that animals less than one month old are more resistant than those two to three months old. The smaller intestinal size of the younger mice must play an important part in this acting as a mechanical barrier to the parasite, since increasing the size artificially by hormone injections was associated with increased susceptibility (Larsh, *ibid.*).

A poor diet, such as bread and water, appears to predispose rats to the mouse strain of this parasite and interfere slightly with the development of age resistance to the rat strain (Shorb, *ibid.*). In a study on concurrent infection, it was found that resistance can be increased markedly in young mice by an infection with a nematode, *Nippostrongylus muris*, when the latter is introduced just prior to the tapeworm (Larsh and Donaldson, 1944).

In addition to the information on natural resistance to *Hymenolepis nana*, considerable data have been obtained from studies on acquired immunity. Numerous workers have shown that the mouse host builds up a strong immunity as the result of an initial infection with this parasite (Grassi, 1887, Joyeux, 1925, Brumpt, 1933, Hunninen, 1935b and Hearin, 1941). Hearin (*ibid.*) presents data showing this active acquired immunity to last as long as 163 days after initial infection and states that it may remain at least 141 days after the removal of the worms. Later it was shown that immunity can be produced artificially by injections of antigen prepared from adult worms (Larsh, 1944a). Hearin also was able to demonstrate clearly that the immunity can be transferred passively by way of immune serum. In other words, he artificially transferred the antibodies present in the serum of immune donors to previously uninfected recipients, the latter when infected later displayed a strong immunity. That such a transfer of protection takes place naturally from immune mothers to offspring was demonstrated by the writer (Larsh, 1942). This study showed what was pointed out above, that passive protection is short-lived, lasting between 37 and 41 days after birth in these experiments. The antibody transfer takes place both *in utero* and *in* the milk, but the latter seems of greater importance. Although the degree of resistance produced was not nearly as high, it was shown later that such a transfer occurs from vaccinated mothers to offspring (Larsh, 1944a).

These studies demonstrated, therefore, that the immunity to this parasite results from the development of humoral antibodies. However these were not demonstrated *in vitro* until later (Larsh, 1943b), when it was found that specific agglutinating, precipitating, and complement-fixing reactions occur.

As pointed out above, there is little evidence to show that the adult stage of

cestodes confers any appreciable degree of immunity to reinfection. This fact has been noted by numerous workers, and was shown by Hearin (ibid.) to be true for the dwarf tapeworm. He introduced the adults into mice by operation, and even those harboring as many as twelve tapeworms for nine days showed no demonstrable active immunity when infected later. Therefore, the results of the studies on *Hymenolepis* bear out the fact already observed, namely that parasitism with the tissue stage of cestodes calls out an active antibody response in the host and accounts, in large part, for the resulting acquisition of immunity.

#### SUMMARY

Brief consideration is given to certain general aspects of immunity included under natural resistance, age resistance, and acquired immunity. This is followed by a discussion of immunity to *Taenia* cestodes, *Echinococcus*, and *Hymenolepis*, giving first a general account of the life cycle followed by selected papers which illustrate the immunity principles involved. It is pointed out that the adult or non-tissue stage of cestodes confers little or no immunity to reinfection, whereas parasitism with the larval or tissue stage elicits an active antibody response in the host which accounts, in large measure, for the ability to resist later infections. These studies agree with the recently accumulated evidence that immunological phenomena among these parasites are similar to, if not identical with, those of many other disease-producing organisms.

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## AVERAGE DIETARY INTAKES IN TWO NORTH CAROLINA RURAL COUNTIES, 1940 AND 1944

By D. F. MILAM, M.D.

*School of Public Health, University of North Carolina*

In January 1940 rural nutrition surveys were begun in North Carolina by the State Board of Health with the purpose of determining the degree of malnutrition that might be present in the state, and as a preliminary to plans for a program of improvement. These surveys were projected in the preceding year and were the natural result of the increasing knowledge of nutrition science, particularly about the vitamins, their chemistry, distribution and essentiality, and particularly as a result of the improved techniques for determining the ill results of nutrition lack in the human physique. For example the nutrient (nicotinic acid or niacin) whose lack leads to the train of events ending in patent pellagra was finally determined in 1937, and astonishing cures of that ailment followed the use of niacin in its treatment. Since pellagra is a chronic disease resulting only from long continued dietary inadequacy, it was natural to conclude that there are conditions preceding the manifest disease where the lack of niacin resulted in ill health short of pellagra and where judicious improvement in diet (or addition of niacin) would break the chain of events leading to the manifest disease. If anything could be done about these "subclinical deficiencies" it seemed to be the obligation of the health authority to explore the possibilities and to take such practical steps as seemed indicated.

Subsequent events have changed the economic status of the very groups where the surveys were projected, and since early 1940 there has developed a tremendous interest in nutrition per se, with a staggering amount of publicity, both commercial and governmental, about diets, vitamins, deficiencies and their cure or prevention. The surveys have therefore not been the study of a fairly stable condition of nutrition or malnutrition, but rather a series of observations on changing food habits. It should be questioned whether these changes were deep and basic or only superficial with additions to the diet in the forms of luxuries that could now be afforded. The surveys have throughout included a detailed record of the actual food items that were eaten by each individual surveyed. Up to the present they have been limited to rural areas, in the belief that malnutrition might be more prevalent there and ordinarily these would be the last areas studied, due to their relative inaccessibility and the scattered distribution of the homes. In the past five years four North Carolina counties have been thus surveyed using a rather exacting procedure that makes use of all the techniques that promised to shed light on the nutrition problem. These included for each individual studied a careful physical examination for any physical signs attributable to dietary lack, a careful dietary record of seven days duration, and the laboratory study of a blood sample for content of vitamins, proteins and minerals. The detailed nature of this procedure limited the extent of the area

that could be covered, but furnished a good opportunity to evaluate the competency of procedures used for the diagnosis of malnutrition.

Numerous reports of the results of these surveys have appeared and are listed in the bibliography at the end of this article. The present report is concerned solely with the diets of two small areas, one in the spring of 1940, the other in the spring of 1944. The period of the North Carolina surveys (1940-44) covers one of improving economic conditions, starting in a year not yet clear of depression factors and continuing to a year of war boom prosperity. It seemed worthwhile to compare the findings in the first and last of the areas surveyed, particularly as regards dietary intake. The diets in 1940 were certainly not built on any great prosperity; those of 1944 were built on a considerably greater cash income. The basic diet of the area might be expected to be largely uninfluenced (in variety) by income, on the theory that people cling to a certain type of diet because they like it. Data on this point might appear in a study of the foods eaten in 1940 and in 1944. With this in mind analysis was made of diets in Chatham County in 1940 and in Alamance in 1944 and results put side by side for study and comparison.

*Description of Areas.* In the spring of 1940 the survey was in a cotton mill village in Chatham County, with a total population of 375, all white. One hundred and sixty-one individual diet records were kept, 37 of these being from adult males. Most of the families had one or more members working in the mill, where maximum wages were approximately \$12.00 per week. A few farmers and business families also lived in the village and were included in the survey. Some mill families had relatives on farms in the region from whom they secured occasional supplies of food. A trucking route from the south passes through the village, and influenced food stocks in the three stores.

In the spring of 1944 the survey was in the northwest quarter of Alamance County, and was composed of a group of rural families selected as a representative sample of the area, geographical, racial, and economic factors being considered. One hundred and fifty-seven white diet records were kept, 36 of these being from adult males. Many of these rural families living towards the center of the county had one or more members employed in a cotton mill. The two small cities in the center of the county constitute an excellent shopping area.

*Methods.* The methods of collecting this dietary data were as follows: A seven-day diet record form was distributed in families, one for each member, this being done at the time of the physical examination in the nutrition clinic. On these forms were recorded for each meal and each between-meal period all the foods eaten and their quantities in ordinary household measurements. This recording was supervised by the nutritionist who during the week made frequent visits to the families when accessible and checked on the completeness and accuracy of the entries. At the time of collecting the record it was checked for accuracy by well directed questions about individual items or omissions and by observations in the kitchen and pantry. The resulting data while not completely accurate still afford a good index of consumption of foods, and on a group basis is a fair index of community nutrition. No better method was deemed

TABLE 1

*Average Daily Intake of Various Foods. Adult White Males in Two North Carolina Counties, Spring 1940 and Spring 1944*

	CHATHAM 1940 37 INDIVIDUALS		ALAMANCE 1944 36 INDIVIDUALS	
	Grams	Ounces (approx.)	Grams	Ounces (approx.)
Cereal Grains				
White Bread	200	6	234	7 $\frac{1}{2}$
Corn Bread	25	$\frac{1}{2}$	15	$\frac{1}{2}$
Cereals	8	$\frac{1}{2}$	16	$\frac{1}{2}$
Dairy Products				
Milk	256	$\frac{1}{2}$ pint	565	1 pint
Cheese	1		5	$\frac{1}{2}$
Ice Cream	28	1	51	1 $\frac{1}{2}$
Fats				
Butter	7	$\frac{1}{2}$	14	$\frac{1}{2}$
Lard	18	$\frac{1}{2}$	16	$\frac{1}{2}$
Other Fats	9	$\frac{1}{2}$	10	
Eggs	70	1 $\frac{1}{2}$ eggs	68	1 $\frac{1}{2}$ eggs
Nuts				
Peanuts	0 6		4 8	$\frac{1}{2}$
Meats				
Lean Pork	29	1	27	1
Fat Pork	12	$\frac{1}{2}$	23	$\frac{3}{4}$
Beef	48	1 $\frac{1}{2}$	16	$\frac{1}{2}$
Fish	9	$\frac{1}{2}$	14	$\frac{1}{2}$
Fowl	12	$\frac{1}{2}$	9	$\frac{1}{2}$
Organ Meats	1		3	
Other Meats	2		4	$\frac{1}{2}$
Vegetables				
Irish Potato	34	1	59	2
Sweet Potato	5	$\frac{1}{2}$	6	$\frac{1}{2}$
Tomato	58	2	43	1 $\frac{1}{2}$
Corn	11	$\frac{1}{2}$	15	$\frac{1}{2}$
Green Leafy Vegetables	16	$\frac{1}{2}$	6	$\frac{1}{2}$
Other Green Vegetables	69	2 $\frac{1}{2}$	62	2
Yellow Veg (Excl Sw. Pot.)	3		12	$\frac{1}{2}$
Peas and Beans (Dried)	7	$\frac{1}{2}$	36	1 $\frac{1}{2}$
Other Vegetables	4	$\frac{1}{2}$	12	$\frac{1}{2}$
Soups	9		24	
Fruits				
Citrus	29	$\frac{1}{2}$ orange	20	$\frac{1}{2}$ orange
Dried	1		10	$\frac{1}{2}$
Other	64	2	17	$\frac{1}{2}$
Sweets				
Molasses	4	$\frac{1}{2}$	1	$\frac{1}{2}$
Cakes and Pies	57	2	70	2 $\frac{1}{2}$
Other Sweets	22	$\frac{1}{2}$	29	1

possible of application and the results by this procedure were considered to be sufficiently informing.

*Findings.* In Table 1 is given the average daily intake of 34 foods by the

white adult males of the two areas. This average is arrived at by totalling all the seven-day intakes of each food item by all the individuals involved, and dividing this by the number of individuals to get the average weekly intake, and by 7 to get the average daily intake. Naturally no individual took exactly this amount of any item, or took all the 34 items. But the average figures do give a fair picture of the community levels of intake of each item. These data are for adult males only, since the age and sex factors greatly modify the amounts of food eaten. In the table the figures are given in grams, and also in ounces, and in one or two places in units such as an egg, a pint, an orange. The common units are added as an aid in visualizing the facts. Many items in the table are of outstanding importance for a good dietary and the North Carolina figures as shown for these two areas are valuable as indications of the quality of diets prevalent here. For example, the consumption of milk, of eggs, of meat and of greens is a matter of concern to every student of American folk diets. In Table 2 is shown the percentages of individuals customarily eating the more important of the food items.

The calculation of the nutrients in these two diets is as follows:

	<i>Chatham County</i>	<i>Alamance County</i>
Calories	2,203	2,615
Carbohydrate	227 gm.	298 gm.
Protein	74 "	88 "
Fat	111 "	119 "
Calcium	0.7 "	1 1 "
Iron	12 mg.	15 mg.
Vitamin A	5,742 Int. Units	5,452 Int. Units
Vitamin C	64 mg.	53 mg.
Thiamin	0.98 "	1 4 "
Riboflavin	1.3 "	2 1 "
Niacin	10.9 "	11 4 "

*Discussion.* In comparing the two areas and their food intakes at the five-year interval, the table is to be interpreted with caution, both as regards the similarities and dissimilarities between the foods eaten by these two population groups at this five-year interval. If the same individuals living in the same area were being compared for dietary change in five years, with the changing economic conditions as the only major factor involved, a little more latitude might be allowed in drawing conclusions, but even here considerable caution would be needed. But in the above we are concerned with totally different families and individuals, living in different counties, working in two separate areas of considerable economic differences, both as to living and working conditions, and in time separated by a five-year interval stretching from the late depression years to the fully developed war boom years. This latter factor may be the major one involved, but it is certainly not the only one.

With this much said it is permissible to state that the diet in 1944 showed considerable improvement on that of 1940, when these two small population groups are used as indices. Twice as much milk and butter were consumed in 1944, and considerably more Irish potatoes. But in 1940 three times as much

beef was consumed as in 1944, a thing which is easily understood by anyone who has seen local shortages under rationing. Nineteen hundred and forty was also superior in consumption of citrus and other fruits, and tomatoes, probably similarly explained, and also more leafy greens were eaten. As explanation of these differences one might cite the improving economic conditions in these five years, but also important would be changing food habits from whatever cause, for example radio propaganda, popular interest in vitamins and foods and their effect on health, as well as difference in social conditions between the two areas.

There is however a striking similarity in the amounts of several important basic foods eaten in the two areas, such as white bread (though in 1944 it was largely enriched), eggs, lean pork, chicken, cooking fats, sweet potatoes, string

TABLE 2  
*Per Cent of Individuals Eating Each of Sixteen Foods of Nutritional Importance*

	CHATHAM SPRING 1940 37 WHITE MEN	ALAMANCE SPRING 1944 36 WHITE MEN
	per cent	per cent
White Bread	100	92
Corn Bread	62	53
Milk	76	97
Ice Cream	19	55
Butter	57	69
Eggs	100	92
Lean Pork	70	78
Fat Pork	68	83
Beef	78	36
Chicken	46	53
Irish Potato	76	97
Tomato	65	78
Green Leafy Vegetables	39	22
Other Green Vegetables	97	95
Citrus Fruits	43	47
Molasses	16	14

beans (including canned), all of them standard articles of diet in good times. The overall picture as revealed by this tabulation of 34 food items is one of striking similarity in the items included in each of these two dietarys; it is one of a quite varied diet with differences in quantities not such as to mark one definitely as good and the other as bad. In fact both diets strike one as quite good and the people getting them as quite fortunate nutritionally. They are a far cry from the reportedly widely prevalent pellagra-producing diet of the first decade of this century.

These, of course, are average diets and give no suggestions of the very few families existing on diets genuinely deficient in one or more of the nutrients. The community picture as here set forth is one of a maintenance diet, fairly adequate even in calories which, though considerably below the Recommended

Dietary Allowances, are still at the level customarily found here for a well fed man of good economic status doing light work.

A word is included about food preferences as given in Table 2. The almost universal consumption of white bread (usually biscuit in rural areas), is not matched by a similar universal consumption of corn bread, which was eaten by only 50 per cent (approximately) of individuals. Milk was a food of prime choice in 1944, 97 per cent of white adults drinking it in the average quantity of one pint daily. Only 75 per cent of the men in 1940 drank milk, and averaged one-half pint. Eggs were nearly a universal choice in both years, average consumption being nearly one and one-half eggs daily. Only 36 per cent reported eating beef in 1944 against 84 per cent in 1940. Approximately 50 per cent ate chicken. Green leafy vegetables were consumed by 40 per cent and 22 per cent of the men in the two years, but other green vegetables (string beans, okra, green peas) were eaten by nearly 100 per cent. Citrus fruits in both years were eaten by approximately 50 per cent of the men. Molasses was in the diet of only 15 per cent of individuals.

The most striking fact about this whole picture is the great variety of foods consumed and the high nutritive value of some of the most commonly used ones. Milk and eggs, lean meat and green vegetables were a staple in nearly all diets. The high nutritive value of collards and turnip greens, of string beans and okra, of tomatoes and sweet potatoes, not to mention those most valuable proteins in eggs and milk, and lean meat, all of these in the proportions as shown in the above tables point to excellent nutritive possibilities. That these were not eaten in adequate quantities by all individuals and in all years goes without saying. That they were eaten when economic conditions warranted is indicated in the above tables, and points to a healthful normal diet in this region. There is hope that the dietary levels set forth above will continue to be the mode in North Carolina, but this is by no means a certainty. If depression comes again, dietary status may well descend with it. Better knowledge of what is an adequate diet may well play an important rôle in reducing the effects of the economic factor. The saving value of a varied diet should have force in depression as well as in prosperity.

At this point it is permissible to state that in prosperity as well as in depression it is well to pay considerable attention to those factors other than nutrition that also play a major rôle in determining physical status, for example, infection and disease, inadequate medical and dental care, inadequate housing, overwork and ill conditions of labor, and many others. A wise social policy aims at alleviating the ill effects of all these social ills by all the power and wisdom we possess. It is not wisdom to neglect nutrition as a factor, but it is also not wise to select mal-nutrition as the sole fount of ill health, to the neglect of other social ills.

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# EXPERIMENTAL DRUG PLANT CULTURE IN NORTH CAROLINA

BY E. A. BRECHT AND H. M. BURLAGE

*School of Pharmacy, University of North Carolina*

Although North Carolina is one of the states leading in the production of crude botanical drugs, it is an interesting fact that this production is dependent upon indigenous plants and that little effort has been made to establish an industry of drug plant cultivation. That drug plant culture is feasible is indicated by the wide variety of useful plants which grow indigenously and the outstanding success in the cultivation of tobacco by methods directly applicable to closely related plants of medicinal value.

The authors have thought for a long time that the cultivation of drug plants might be advantageous to the state of North Carolina by increasing both the diversification of crops and the farm revenue. The World War II gave added incentive for this work by eliminating the source of supply for many important drugs.

In this modest test of drug culture possibilities, it was decided to concentrate the effort on a few well chosen representatives of botanical drug plants. Most of the work was done during the summer of 1942 on the farm of the second author located two miles northeast of Chapel Hill.

## BELLADONNA

*Atropa Belladonna* L. (Fam. Solanaceae) yields two drugs, the leaf and the root. This plant was considered a likely prospect because it is related to tobacco and because the war had cut off the supply from central Europe, causing a rise in the pound price from 15¢ and 11¢ in 1937 to \$2.25 and \$2.00 in 1941 for the leaf and the root, respectively (Crooks, 1941).

Eight grams of seed were obtained from Dr. E. B. Fischer, Professor of Pharmacognosy at the College of Pharmacy, University of Minnesota. Half of the seed were treated with concentrated sulfuric acid to speed germination, and the other half were planted without treatment as a control. No difference was detected in the two flats of seedlings. In May, 987 seedlings were set out, divided into four plots of ground varying chiefly as to drainage. About eighty per cent survived the transplanting, but later alternating floods and heat and drought killed off all but 44 plants by September when the leaves were harvested. From these plants 325 gm. of leaf, after drying, was obtained. On this basis, with complete survival of plants, it might be expected that an acre of land would produce about 200 pounds of dried belladonna leaf. The dried leaf, including stems less than 10 mm. in diameter, was assayed according to the Pharmacopoeial method, and was found to contain 0.60 per cent of alkaloids. This quality is twice the required official minimum of 0.3 per cent alkaloids. (The stems over 10 mm. diameter were separately dried and assayed. They contained only 0.07 per cent alkaloids.) The roots were not collected, because it was considered desirable to

determine whether they could survive the winter and produce a larger crop. Only one root survived. It is now known that belladonna survives the winter only if considerable portions of the stem are left intact (Sievers and Lowman, 1944). During 1944, twelve grams of seed were obtained from the surviving plant, and these will be planted later.

#### SPIGELIA

*Spigelia marilandica* L. (Fam. Loganiaceae) yields a drug consisting of the dried rhizome and roots. Other names for this drug are Pink Root and American Worm Root. Youngken, 1943, states: "Pink root was formerly collected in large amounts in North Carolina and Tennessee but is becoming very scarce in these states at present. Most of the commercial article is now gathered in Mississippi. . . . The most common substitute and adulterant has been the rhizome and roots of *Ruellia ciliosa* Pursh. (Fam. Acanthaceae), commonly known as East Tennessee Pink Root, a perennial herb, somewhat resembling Spigelia and often found growing in vicinities where the latter abounds."

Spigelia is used in proprietary preparations for an anthelmintic, especially in ascarides. Interest in this plant was twofold: to test the practicability of producing the root commercially and to produce the drug free from Ruellia.

Two lots of 1,000 Spigelia (?) plants were obtained from Mr. J. D. McIntyre, president of Dr. D. Jayne and Son, Inc., one lot in 1942 and the other in 1943. Both lots contained considerable Ruellia, which was eliminated when the plants flowered. The plants grew satisfactorily. At present, attention is centered on increasing the holdings of identified stock. This is being done by root division and from the seeds.

#### OTHER PLANTS

Smaller plantings of other plants were made to test the potentialities of production. In general the plantings of 1942 were adversely affected by an early drought soon after transplanting, later by dry heat, and, finally, by a flood of the low land where the plantings were made. Although final decisions cannot be made, it seems to be indicated that Digitalis, Valerian, and Castor are not good prospects for cultivation in this area, while Sage, *Saponaria officinalis*, Aletris, and Helonias, all of good quality, can be cultivated to advantage.

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# THE ASSAY OF NUX VOMICA PREPARATIONS BY CHROMATOGRAPHIC METHODS<sup>1</sup>

By JOSEPH LAROCICA<sup>2</sup> AND HENRY M. BURLAGE

*School of Pharmacy, University of North Carolina*

WITH TWO FIGURES

Analysis by chromatographic adsorption was first proposed in 1906 by M. Tswett, a botanist, who was able to resolve the petroleum extract of dried leaf material into five different components using precipitated chalk (precipitated calcium carbonate) as an adsorbent. Since that date, a number of workers have employed the method and in 1931, Kuhn and Lederer were able to obtain alpha- and beta-carotene from carrot root by the procedure.

From 1935 to 1940 some work was done on the analysis of the alkaloids by this method and it was first applied by Valentin (1937) to pharmaceutical preparations including an alcoholic solution of Balsam Peru and to Tincture of Digitalis. Kundo (1937) separated morphine and thebaine (1% each) in acetone solution by using alumina as the adsorbent and benzene as the eluant. Spath and his coworkers (1937) isolated hydroxysanguinarine from *Sanguinaria canadensis* by chromatographing the chloroformic extract of the drug on an alumina column. In 1940, the chromatographic separation of morphine, codeine, narcotine, and papaverine was accomplished by Levi and Castelli.

In 1937, Merz and Franck isolated, quantitatively, cantharidin from Tincture of Cantharides. The same year, Ernst and Weiner, by chromatography, extracted and separated the anthraquinones and anthrols from the anthraquinone drugs including Aloe, Cascara Sagrada, Rhamnus Catharticus, Senna, etc.

Valentin and Franck (1936), in addition to developing a quantitative procedure for the determination of alkaloids in Tincture of Belladonna, Infusion of Coffee, and Extract and Fluidextract of Cinchona, also described a chromatographic procedure for the quantitative determination of the total alkaloids in Tincture of Strychnine. It was this procedure that led to the problem under investigation in this report. These workers obtained excellent results by their procedure and these have been substantiated by the present investigation.

Since such excellent results were obtained in the determination of the total alkaloids and since a separation of isomeric compounds has been accomplished, it was deemed advisable to attempt a separation of strychnine and brucine so that a simple quantitative procedure for strychnine, the active constituent in the preparations of Nux Vomica, might be devised. Furthermore, the present official method<sup>3</sup> for the determination of strychnine in the Tincture of Nux Vomica depends upon the oxidation of the brucine by a nitric acid-sodium nitrite

<sup>1</sup> A portion of a thesis presented in partial fulfilment of the requirements for the M. S. degree in Pharmacy, June, 1944

<sup>2</sup> American Pharmaceutical Association Research Fellow, 1943-4.

<sup>3</sup> The Pharmacopoeia of the United States of America. Twelfth Revision, p 515.

mixture, presumably without the destruction of any of the strychnine. The period of time allotted for the oxidation is open to question and apparently this procedure has not been found to be entirely reliable as is shown by the number of times it has been accepted and rejected as an official method of assay. Other objections to the present method of assay are (1) the cost of reagents, (2) attention and care required throughout, and (3) the time required to carry out a single set of determinations.

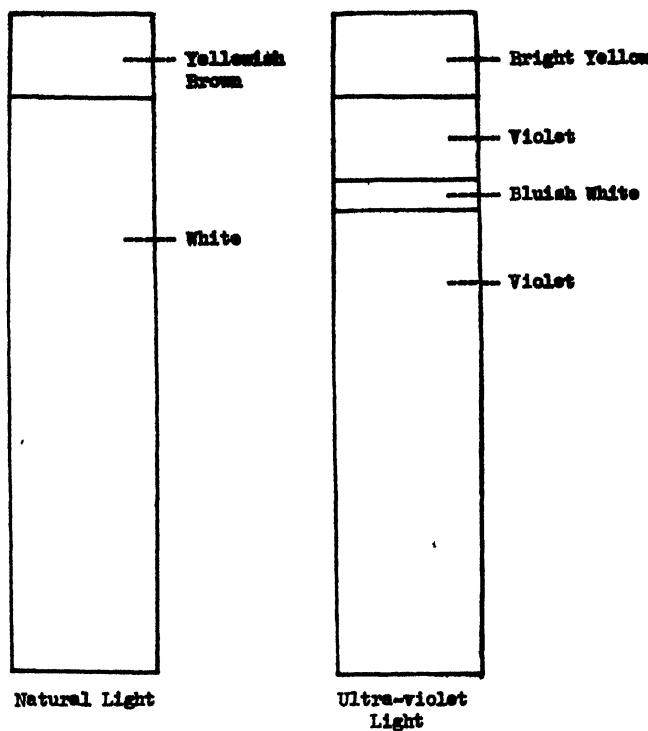


FIG. 1 The appearance of a Chromatogram of Tincture of Nux Vomica in natural and ultra-violet light

#### EXPERIMENTAL

##### *A. The Chromatogram of the Alkaloids of Nux Vomica under Ultra-violet Light*

Since strychnine and brucine are colorless compounds, the adsorption bands are not visible to the naked eye; both compounds, however, exhibit fluorescence under the influence of ultra-violet light.

A chromatogram of Tincture of Nux Vomica (10 cc.) on an alumina<sup>4</sup> in a quartz tube was observed under ultra-violet light. The appearance of the column in ordinary light and under ultra-violet light is shown in Figure 1. This coincides with the observations of Valentin and Franck.

<sup>4</sup> Activated alumina, 200 mesh, marketed by the Alumina Ore Company, East St. Louis, Illinois.

Attempts to separate the two alkaloids in the tincture by elution of the alumina column with alcohol (70% by volume), followed by subsequent sectioning of the column under the light, were not successful.

### B. Separation of the Alkaloids by Destruction of the Brucine

Attempts to destroy the brucine by the usual oxidation procedure on columns of alumina, kaolin (1 part of asbestos to 3 parts of kaolin), activated bentonite (1 part of asbestos to 3 parts of the clay), Fuller's earth, and infusorial earth, followed by elution of the oxidation products, or elution of the strychnine, were unsuccessful. This was due to (1) the action of the oxidizing mixture on the column itself (alumina), (2) the interference of the products of oxidation which readily eluted along with the strychnine (kaolin and activated bentonite), and (3) the lack of adsorption of the alkaloids (Fuller's earth and infusorial earth).

### C. Separation of the Alkaloids by Elution with Suitable Solvents

The simplest method for this type of separation of strychnine and brucine would be the elution of one or the other alkaloid from the column by means of a selective eluant. A search was conducted to ascertain an eluant which would carry the alkaloid, preferably strychnine, into the filtrate and leave the other one on the column. This search showed that, although brucine is generally more soluble than strychnine, it is eluted with more difficulty than the latter probably because of the presence of methoxy groups.

In this portion of the study, a stock solution was prepared containing in each 100 cc. of 70% alcohol, 0.1163 gram of strychnine and 0.1147 gram of brucine, the ratio of the two alkaloids in the seeds of *Nux Vomica*.<sup>5</sup> Five cubic centimeters of this solution were chromatographed on a column of alumina 15 centimeters long and one centimeter in diameter and then eluted with various solvents. The progress of the elution was followed by subjecting the filtrate at various intervals to the *Fading Purple Test* for the detection of strychnine and to the *Nitric Acid Oxidation Test* for brucine.<sup>6</sup>

On the basis of the results obtained, the eluants tested may be classified into the following groups:

#### Group I. Eluants which eluted both alkaloids simultaneously and completely

1. Methyl alcohol	6. Tetrahydrofurfural
2. Ethyl alcohol	7. Ethylene dichloride
3. Ethyl acetate	8. Perchlorethylene
4. 2-Methyl pentadiene	9. Amyl acetate
5. n-Amyl alcohol	10. Benzene
11. Dichlorethyl ether	

#### Group II. Eluants which eluted all of the brucine and a part of the strychnine

1. Butyl acetate	2. Isopropyl ether
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<sup>5</sup> Winterstein, E., and G. Tier, "Die Alkalioide," Berlin, 1931, p. 445.

<sup>6</sup> Henry, T. A. "The Plant Alkaloids," p. 507 P. Blakiston's Son and Co., Inc., Philadelphia, 1939.

*Group III. Eluants which eluted all of the strychnine and a part of the brucine*

1. Chloroform
2. Petroleum ether (30-60°C. boiling range)
3. Chloroform-Petroleum ether (1:10)
4. Chloroform-70% alcohol (3:10)
5. Chloroform-70% alcohol (2:10)

*Group IV. Eluant which eluted all of the strychnine and none of the brucine*

1. Ligroin (90-110°C. boiling range)

In the latter case, as the ligroin passed through the column, samples of the filtrate were taken periodically and tested for the two alkaloids. The elution was stopped when the filtrate no longer gave a positive strychnine test. The column was then removed and the alkaloids were extracted from it with chloroform. The chloroformic solution gave a positive test for brucine but a negative one for strychnine, indicating that all the strychnine had been eluted. The filtrate was evaporated to one-fourth volume and the concentrated liquid yielded

TABLE I  
*The Elution of the Chromatogram of a Stock Solution with Ligroin*

AMOUNT OF FILTRATE	STRYCHNINE TEST	BRUCINE TEST	AMOUNT OF FILTRATE	STRYCHNINE TEST	BRUCINE TEST
cc.			cc.		
10	Pos.	Neg.	70	Pos.	Neg.
20	Pos.	Neg.	80	Pos.	Neg.
30	Pos.	Neg.	90	Pos.	Neg.
40	Pos.	Neg.	100	Pos.	Neg.
50	Pos.	Neg.	110	Pos.	Neg.
60	Pos.	Neg.	120	Neg.	Neg.

a positive strychnine test and a very faint one for brucine. Table I shows the progress of this elution.

In the next series the length of the column was reduced from 15 centimeters to 10 centimeters and the results are shown in Table II.

The results indicate that, in addition to the eluant used, the length and diameter of the column has a definite effect upon the selective elution of the adsorbed alkaloids. In this case reducing the column lessens the possibility of eluting brucine.

*D. Sensitivity of the Qualitative Tests Used*

Since the tests for elution were of a qualitative nature, and since no exact figures concerning the sensitivity of the two qualitative tests employed are available, it was deemed advisable to determine this quantitatively.

It was found that the *Fading Purple Test* will detect strychnine in a solution of 1:500,000 and the *Nitric Acid Oxidation Test* will detect brucine in a solution 1:175,000. An attempt to establish the colors given by samples of the various dilutions by means of the Munsell System of Color Notation was not possible

in the case of the *Fading Purple Test* due to the rapid disappearance of the color produced.

However, it was found possible to standardize the colors produced by the test for brucine. These results are shown in Table III.

TABLE II

*The Elution of the Chromatogram of a Stock Solution with Ligroin*

AMOUNT OF FILTRATE	STRYCHNINE TEST	BRUCINE TEST	AMOUNT OF FILTRATE	STRYCHNINE TEST	BRUCINE TEST
cc.			cc.		
10	Neg.	Neg	50	Pos.	Neg.
20	Pos.	Neg.	60	Pos.	Neg.
30	Pos.	Neg.	70	Pos.	Neg.
40	Pos.	Neg.	80	Neg.	Neg.

TABLE III

*Sensitivity of the Nitric Acid Test for Brucine*

DILUTION	BRUCINE	MUNSELL COLOR	DILUTION	BRUCINE	MUNSELL COLOR
	Gms /cc			Gms /cc	
1:20,000	0 000057	Moderate Orange	1:100,000	0 000014	Moderate Yellowish Orange
1:30,000	0 000038	Moderate Orange	1:125,000	0 000009	Weak Yellowish Orange
1:40,000	0 000028	Moderate Orange	1:150,000	0 000007	Weak Yellowish Orange
1:50,000	0 000023	Moderate Yellowish Orange	1:175,000	0 000006	Weak Yellow
1:75,000	0 000015	Moderate Yellowish Orange	1:200,000	0 000005	Colorless

#### *E. Description of the Apparatus*

The apparatus used in the experiments is shown in Figure 2, and, it may be noted, consists of materials which are readily available in most analytical laboratories.

#### *F. The Procedure*

The column is prepared by inserting a cotton plug in the tube over the cork at the lower end. Alumina is packed into the tube by tapping it gently against a hard surface so that the final length of the adsorbing column is 30 centimeters. Uniform but not too tight packing is essential.

The sample (15 cc.) is added to the column through the separatory funnel and gentle suction is applied to the system by means of a filter pump until the pressure is reduced to approximately 500 millimeters of mercury. When the entire sample to be tested is passed into the column, the ligroin, which has been purified by double distillation over sodium hydroxide to remove acid polymers that might be present, is added before the column becomes dry and the elution is continued

until 200 cubic centimeters have passed into the column. The elution is stopped as the last portion of the eluant disappears into the column at the top and the column is *not* completely drained. The volume of the filtrate is approximately 180 cubic centimeters.

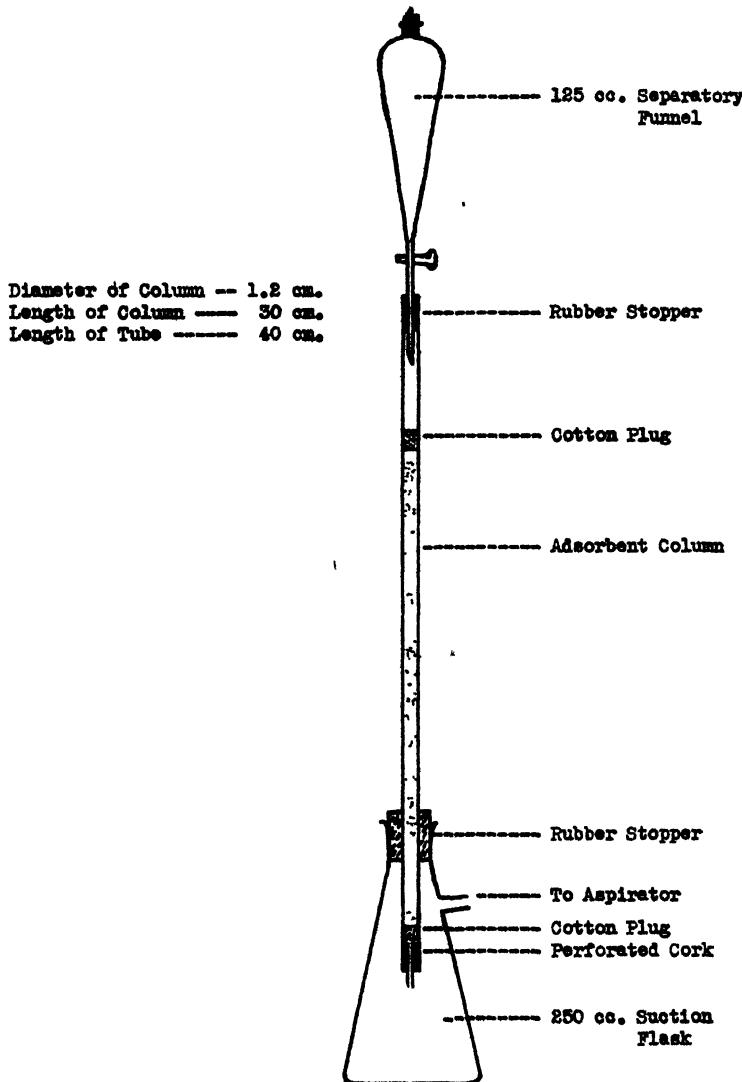


FIG 2 Apparatus for chromatographic adsorption  $\times \frac{3}{4}$

The filter flask is removed and equipped with a capillary tube and the filtrate evaporated to dryness under reduced pressure at the temperature of the water bath. Added to the residue, which may have a yellowish tinge due to the presence of the ligroin but which seems to have little or no effect on the results, are 35 cubic centimeters of N/50 sulfuric acid, accurately measured, and 10 cc. of

distilled water. The solution is heated until all of the alkaloid is dissolved and then cooled to room temperature. It is then titrated with N/50 sodium hydroxide solution measured from a microburette, using methyl red test solution

TABLE IV  
*Analysis of Tincture of Nux Vomica*

SAMPLE	TOTAL ALKALOIDS CHROMATO- GRAPHICALLY	TOTAL ALKALOID U.S.P. X METHOD	STRYCHNINE CHROMATO- GRAPHICALLY	STRYCHNINE U.S.P. XII METHOD	STRYCHNINE COMMERCIAL ANALYSIS
	Gms./100 cc.	Gms./100 cc.	Gms./100 cc.	Gms./100 cc.	Gms./100 cc.
1	0.2806	0.2579	0.1199	0.1153	
2	0.2806	0.2809	0.1199	0.1166	
3			0.1145	0.1131	
4			0.1145	0.1125	
Aver.	0.2806	0.2594	0.1172	0.1144	0.1198

TABLE V  
*Analysis of Fluidextract of Nux Vomica*

SAMPLE	TOTAL ALKALOIDS CHROMATO- GRAPHICALLY	TOTAL ALKALOIDS U.S.P. IX METHOD	STRYCHNINE CHROMATO- GRAPHICALLY	STRYCHNINE N.F. VII METHOD	STRYCHNINE COMMERCIAL ANALYSIS
	Gms./100 cc.	Gms./100 cc.	Gms./100 cc.	Gms./100 cc.	Gms./100 cc.
1	2.4825	2.4679	1.1592	1.1457	
2	2.4825	2.4873	1.1592	1.1390	
3			1.2885	1.0600	
4			1.1236	1.0633	
Aver.	2.4825	2.4776	1.1826	1.1020	1.2160

TABLE VI  
*Analysis of Extract of Nux Vomica*

SAMPLE	TOTAL ALKALOIDS CHROMATO- GRAPHICALLY	TOTAL ALKALOIDS U.S.P. X METHOD	STRYCHNINE CHROMATO- GRAPHICALLY	STRYCHNINE N.F. VII METHOD	STRYCHNINE COMMERCIAL ANALYSIS
	Gms./100 cc.	Gms./100 cc.	Gms./100 cc.	Gms./100 cc.	Gms./100 cc.
1	17.097	17.104	7.530	7.442	
2	17.097	16.901	7.530	7.495	
Aver.	17.097	17.003	7.530	7.469	7.637

as the indicator. Each cubic centimeter of N/50 acid is equivalent to 0.006688 gm. of strychnine.

#### *G. Quantitative Determination of the Preparations of Nux Vomica*

The quantitative determination of strychnine in these preparations<sup>7</sup> was conducted chromatographically and by the official procedure. In addition, the total alkaloidal content was determined by the Valentin-Franck method and by

<sup>7</sup> The samples of the tincture, the fluidextract, and the extract, and the assay data for these samples were generously supplied by Eli Lilly and Co., Indianapolis, Indiana.

former official methods. All values are given in terms of grams of alkaloids per 100 cc. of sample.

1. *Commercial Tincture.* Fifteen cubic centimeters of the tincture were treated and tested as in Part F. The results are shown in Table IV.

2. *Commercial Fluidextract.* For this determination a solution was prepared by diluting 5 cubic centimeters of the fluidextract to 100 cc. with 70% alcohol and 15 cubic centimeters of this solution used. The results are shown in Table V.

3. *Commercial Extract.* For the assay, a solution was prepared by dissolving an accurately weighed sample of about 1.5 gm. of the extract in 100 cubic centimeters of 70% alcohol. Fifteen cubic centimeters of this solution were assayed chromatographically. The results are given in Table VI.

#### CONCLUSIONS

1. By using ligroin as an eluant and alumina as the adsorbent, it is possible to effect a separation of strychnine and brucine from the preparations of Nux Vomica by continuous elution.

2. The sensitivities of the *Fading Purple Test* for strychnine and the *Nitric Acid-Oxidation Test* for brucine have been found to be 1:500,000 and 1:175,000, respectively, and the color values of the latter test with brucine solutions at various dilutions have been determined by the Munsell Color Notation.

3. No elaborate apparatus is necessary to conduct the proposed analysis.

4. The results obtained by the proposed procedure were, in general, higher than those obtained by the official methods. The lower results of the official methods may be attributed to a possible loss of alkaloids during the extraction and purification processes, which are eliminated by the proposed procedure, to the possibility of some decomposition of the strychnine by the oxidizing mixture, and to the experience of the operator.

5. The proposed method of assay appears to be more accurate and, in the case of the tincture and the fluidextract, the results reveal an error of 0.3% if the commercial analysis submitted is assumed to be 100% correct.

6. The proposed procedure is more economical from the standpoint of time and the amounts of reagents required.

7. It is recommended that the proposed method be given consideration for adoption as the official method for the assay of strychnine in the preparations of Nux Vomica.

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# A COMPARATIVE STUDY OF TOLUENE AND XYLENE IN THE DETERMINATION OF MOISTURE IN VEGETABLE DRUGS BY THE DISTILLATION METHOD<sup>1</sup>

BY CYRIL K. WHEELER, JR., AND MARION L. JACOBS

*School of Pharmacy, University of North Carolina*

WITH THREE FIGURES

## INTRODUCTION

Moisture is ordinarily considered as being held in organic materials much in the same way as water is held in a wet sponge. The removal of moisture from such materials has occasioned much difficulty in the past. Furthermore, the moisture content of crude vegetable drugs is somewhat variable, since most of these drugs are more or less hygroscopic. It has been found that some drugs take up as much as five per cent of moisture based on their air-dried weight when stored in a humid atmosphere, and that they lose corresponding amounts of water when stored in a dry atmosphere. Generally, assays are made to determine the amount of active constituent contained in a drug in the condition in which it is purchased or used. In accurate scientific work, however, and in those cases where the drug is to be sold with a guaranteed assay, the per cent of active constituent must be calculated upon the basis of the moisture-free drug.

The method most commonly followed in determining the moisture content of a vegetable drug is to heat at 100°C. in an oven until the weight becomes constant. Since, however, most vegetable drugs contain variable amounts of volatile substances other than water, such as essential oils, ethers, esters, amines and alkaloids in some instances, this method is not generally applicable except for rough estimations. When the drug contains volatile matter other than water, the *United States Pharmacopoeia XII* and the *National Formulary VII* directions require that the volatile ether-soluble extractive shall be determined and the weight of the latter subtracted from the weight lost by the drug upon drying, the difference to be taken as the moisture content of the drug.

A somewhat more satisfactory method for the determination of moisture in vegetable drugs is the distillation method. This method was made official in the Eleventh Revision of the *United States Pharmacopoeia* for the first time, under the title of "Moisture Method by Toluene Distillation," and has been retained in the Twelfth Revision without much change.

The purpose of this investigation has been to determine which liquid, toluene or xylene, gives better results when used as the distilling liquid.

The procedure followed was that given in the *United States Pharmacopoeia*, Twelfth Revision, on page 629. However, before giving the experimental data,

<sup>1</sup> This paper is based on a thesis presented to the Graduate School of the University of North Carolina by Cyril K. Wheeler, Jr. in partial fulfillment of the requirements for the degree of Master of Science.

it would seem desirable to discuss briefly the method, as well as the apparatus and solvents used, from a historical standpoint.

#### GENERAL

The determination of moisture by distillation methods appears to have been suggested by Hoffman (1) and Sjollema within the same year, 1901. Hoffman,

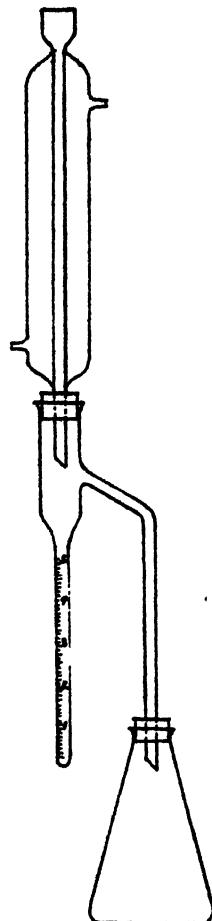


FIG. 1. Assembled apparatus for moisture determination by the distillation method

working in Germany, suggested it as a means of determining the moisture content of grains and received a patent on the process in 1901, while Sjollema, working in Holland, suggested it as a means of determining water in foodstuffs. The determination of water in tars by direct distillation had been previously performed, but it was Jayne (2) of this country who in 1903 suggested the addition of a hydrocarbon boiling above 100°C. to the tar before distillation as a more rapid means of determining the water content. Marcusson (3) in 1904 determined the water content of lubricating greases by distillation with toluene, and

later in 1905 extended the method to include oils, fats, soaps, resins, etc., using water-saturated xylene in place of toluene. The sample was introduced into a flask containing a measured quantity of xylene, and the moisture was distilled into a receiver constricted at the bottom to a calibrated tube until several hundred cubic centimeters passed over. The column of water was then measured. The simple distillation method, often referred to as the Hoffman-Marcusson method, with variations has been used with varying degrees of success on a variety of materials.

The method of Marcusson was greatly improved in 1920 by Dean and Stark (4), who devised an apparatus whereby the sample was refluxed with a liquid immiscible with water, the receiver being essentially a calibrated sedimentation tube with a side arm, which returns the medium to the flask, while the water is trapped by dropping to the bottom of the calibrated tube.

The Dean and Stark apparatus has been approved by the Association of Official Agricultural Chemists and by the Institute of Petroleum Technologists for use in the tentative method for determining moisture, following a report upon the apparatus in 1928 by Bidwell and Sterling (5), whose sole innovation consisted in using a collecting tube of narrower diameter, thereby gaining greater accuracy of measurement. The apparatus recommended in the Twelfth Revision of the *U. S. Pharmacopoeia* is Bidwell and Sterling's Modification of the Dean and Stark apparatus (Figure 1).

#### APPARATUS

Over twenty-five different modifications of apparatus have been proposed for carrying out the determination of water by distillation methods, each with some supposed advantage over its predecessor, or for some special adaptation of the method.

Early workers felt that metallic stills were of distinct advantage because of the danger of fire. Hoffman (1), Jayne (2), Schwalbe (6), von Haydin (7), and others used metal stills. However, since the advent of pyrex glassware, glass has been the more acceptable on account of its transparency.

The early workers used a simple distillation apparatus. Among those using an inclined condenser were Jayne (2), Marcusson (3), Dean (8), Somner (9), and Mai and Rheinberger (10). Those using a vertical condenser were Hoffman (1), Aschman and Arend (11), Brown and Duval (12), Michel (13), von Haydin (7), Schlaepper (14), Arragon (15), and Merl and Reuss (16).

Some workers used a cooled receiver as a condenser. Among these were Schwalbe (6), Thoemer (17), and Rusche (18).

Special apparatus was designed by Gray (19), Besson (20), Kingman (21), Kattwinkel (22), Pritzker and Jungkunz (23), and Tausz and Rumm (24), from which evolved the reflux distillation method.

The original method of Marcusson was greatly improved by Dean and Stark, whose apparatus resembled that of Pritzker and Jungkunz in its essentials, but possessed the great advantage that the water was collected in a tube away from

the main stream of returning liquid. The collecting tube could, therefore, be made of any dimensions in accordance with the accuracy of measurement required. The tube proposed by Bidwell and Sterling is identical in principle with the apparatus used by Dean and Stark. They found that by reducing the diameter of the calibrated receiving tube the column of water could be read with sufficient accuracy for a wide variety of products. A calibrated section taken from a 5 cc. Mohr pipette, sealed at one end and attached to the apparatus served very well for the purpose.

Other reflux adaptations have been used by Liese (25), Normann (26), Herbst (27), Aufhauser (28), Schaefer (29), Kreis (30), and Dedlow and Smith (31).

The method has always been open to the criticism that a ring of water-drops tends to collect in the condenser tube above the vapor. Many attempts have been made to eliminate this cause of error by modifications in the design. Normann (26), Pritzker and Jungkunz (23), Schaefer (29), Boller (32), and Lundin and Lundin (33) have made suggestions as to how this might be overcome. Jones and McLachlan (34) recommend the removal of water droplets from the condenser by means of a copper-wire spiral.

#### DISTILLING LIQUID

The distilling liquid or "Arbeitsflüssigkeit" as Hoffman designated it, has, with few exceptions, been a hydrocarbon. In place of turpentine, as used by Hoffman, or toluene, or xylene, other hydrocarbons and hydrocarbon derivatives have been used. Inasmuch as xylene has been used more than other hydrocarbons, the method has often been referred to as the xylene method for moisture determination. The American Chemical Society in its standard method for the analysis of commercial fats and oils has adopted the distillation method with the use of acetylene tetrachloride as the distilling liquid. *The United States Pharmacopoeia XII* uses toluene as the distilling liquid at the present time.

Other liquids which have been used for the purpose are: amyl acetate, amyl acetate and amyl valerate, benzene, petroleum ether, cleaner's naphtha, coal tar naphtha, cumene, cymene, gasoline, heptane, kerosene, liquid paraffin, liquid paraffin and gasoline, liquid paraffin followed by turpentine oil, limonene, lubricating oil followed by turpentine oil and five per cent toluene, perchloroethylene, pinene, toluene and benzene, toluene and xylene, water-saturated toluene, water-saturated xylene, water-saturated toluene and benzene, xylene and benzene. Most of the above liquids have been found unsatisfactory and are not recommended by the workers who used them.

The merits of these distilling liquids may be considered from several points of view. It would seem, first of all, that a definite chemical compound would naturally be more desirable than a mixture that varies in composition. This holds true not only as to boiling liquid temperature and solubility of water in the distilling liquid, but also when the method is used for moisture determination of aromatic and other drugs, e.g., or for a preliminary investigation of drug constituents soluble in the distilling liquid. These considerations are against the

use of such mixtures as petroleum products in general, though in specific cases such hydrocarbons may have the advantage of cheapness in technical determinations. Even commercial xylene, an indefinite mixture of three isomeric xylenes, shows certain disadvantages in this respect.

Several workers feel, however, that a mixture of liquids insures all the water being driven over—the liquid boiling just above 100°C. carrying over most of the water and the high boiling liquid insuring the water being swept out of the condenser into the receiver. Hoffman used 5 per cent toluene with turpentine oil in order to get the last traces of water out of the product. Schwalbe in working with cellulose materials found that a toluene-benzene mixture of b.p. 105°C. was not efficient in this respect. Prutzman determined water in petroleum using a benzene and toluene mixture. He points out that, on distillation, the benzene and water are first driven over, and then the toluene sweeps out the condensed water clinging to the sides of the apparatus. Scholl and Strohecker recommend xylene with 5 per cent toluene for the determination of water in spices. This mixture gives a slow rise in temperature between 135 and 139°C.

Dean and Stark recommend several mixtures which produce a gradual rise in temperature, but in their reflux modification of the method they accomplish several things not accomplished by the simple distillation method. For instance, Marcusson and many later workers found it necessary to pre-saturate the distilling liquid with water so that it would take up none from the material under examination, but in the reflux method only a few cubic centimeters of hydrocarbon are in contact with the water, and it is found essential to pre-dry the distilling liquid before using in order that none of the water measured has come out of the distilling liquid.

It is obvious that the distilling liquid must be chemically inert in its reaction towards water, another point which would favor a definite chemical rather than mixtures, the composition of which is not always known.

The boiling temperature naturally is an important factor in many cases. If changes need not be feared, the higher boiling hydrocarbons may be regarded as more satisfactory than the lower boiling ones, especially in the matter of reducing the time of an analysis. As early as in 1916, Folpmers attributed high results obtained with xylene in the analysis of spices to decomposition, which did not occur with either benzene or toluene. Hilts, in 1922, in working with fruits, found distillation with xylene decidedly limited, as levulose decomposes above 70°C. Bidwell and Sterling (5), in 1925, also showed that xylene decomposes levulose with the formation of water and that toluene does not. Normann (26) recommends toluene or benzene as the ideal distilling liquid for the determination of water in anything liable to undergo decomposition. Tate and Warren (35) state that the use of xylene did not appeal to them on account of the mutual solubility of xylene and water, while the higher boiling point rendered it very liable to decompose the substance under examination.

Most of the work on moisture determinations has been done with a free flame, although it is advisable to use a bath or hot plate, especially for samples containing sugar, to prevent charring.

## CONCLUSIONS

The following conclusions can be made after having reviewed the literature on the determination of moisture by distillation methods:

1. The best apparatus to use is that designed by Dean and Stark with the modifications of Bidwell and Sterling (Fig. 2).

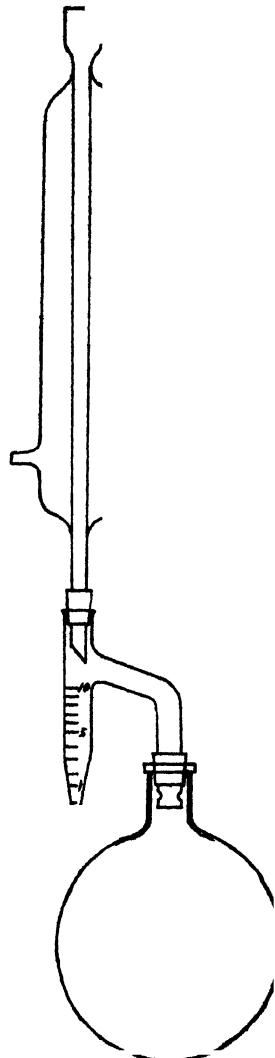


FIG. 2. APPARATUS OF DEAN AND STARK

2. Evidence has been presented that would indicate the distilling liquid should be a definite chemical compound (hydrocarbon) and not a mixture.
3. The boiling temperature should be high enough to carry over all the water in a reasonable time, but not so high as to decompose the substance with the production of water.

4. The liquid should be lighter than water, and their mutual solubility should be negligible for all practical purposes.

#### EXPERIMENTAL

*Purpose.* Many materials have been examined for their moisture content by the distillation method with varying results, depending, in most cases, on the liquid used. It is obvious that some liquid should be adopted in an official method that would insure a yield of water comparable to other methods of determination, and at the same time would be applicable to the determination of moisture in all members of the group. For example, a liquid should be chosen for determining moisture in a vegetable drug which would drive off as much water as the oven method and could also be used without reservation for all vegetable drugs.

The only way to determine which liquid to use for a certain group of substances such as the vegetable drugs, is to run a series of moisture determinations with many members of the group, using liquids which have given good results with other materials and which possess certain essential properties. As has been pointed out, toluene and xylene have been the most largely used liquids in the distillation method. Although toluene is the liquid used in the method official in the *United States Pharmacopoeia XII*, many investigators have shown that better results (that is, higher yields of water without evidence of decomposition) could be obtained with xylene. These investigators, however, have limited themselves to determining the moisture content of only one or two drugs at a time and comparing their results with those of earlier workers.

It is the purpose of this investigation, therefore, to carry out a series of moisture determinations on a sufficiently large number of vegetable drugs to be able to reach, if possible, definite conclusions as to the relative merits of the distilling liquids toluene and xylene.

*Selection of Drugs.* The vegetable drugs used in the experiments were selected with the view of having all parts of a plant represented. Following is a list of the drugs used: Aconite U.S.P., No. 40 powder; Agar U.S.P., shreds; Aloe U.S.P., No. 40 powder; Althea U.S.P., cut; Anise N.F., whole; Aspidium U.S.P., whole; Belladonna Leaf U.S.P., No. 60 powder; Cascara Sagrada U.S.P., ground; Chondrus N.F., whole; Cinchona U.S.P., No. 40 powder; Cinnamon U.S.P., No. 40 powder; Clove U.S.P., whole; Colchicum Corm N.F., No. 60 powder; Colchicum Seed U.S.P., No. 40 powder; Digitalis Leaves U.S.P., granulated; Ergot U.S.P., granulated; Eriodictyon U.S.P., No. 40 powder; Eucalyptus Leaves, No. 20 powder; Gelsemium N.F., No. 40 powder; Gentian U.S.P., No. 40 powder; Ginger U.S.P., No. 40 powder; Glycyrrhiza U.S.P., No. 20 powder; Hyoscyamus U.S.P., No. 40 powder; Kino U.S.P., granulated; Myristica U.S.P., No. 50 powder; Myrrh U.S.P., No. 40 powder; Nux Vomica U.S.P., No. 40 powder; Orris Root N.F., powdered; Peppermint U.S.P., broken leaf; Pomegranate, No. 20 powder; Physostigma Venenosum, ground; Quassia N.F., cut; Quillaja N.F., No. 60 powder; Rhubarb U.S.P., No. 40 powder; Senna U.S.P., granulated; Spearmint U.S.P., broken leaf; Squill U.S.P., granulated; Traga-

canth U.S.P., ribbons; Trifolium N.F., No. 40 powder; and Wild Cherry U.S.P., ground.

It is noted that a total of forty vegetable drugs of varying degrees of subdivision are used in the experiments.

*Procedure.* The procedure as outlined in the *United States Pharmacopoeia XII* was followed. A 500 cc. Erlenmeyer flask of resistance glass, a sealed-in, straight-tube, Liebig condenser, about 500 millimeters in length and a Dean and Stark distilling tube receiver were used.

Fifty grams of the vegetable drug were accurately weighed and placed in a flask. Sufficient liquid was added to cover the drug completely and the apparatus was connected. The receiving tube was filled with liquid by pouring it through the top of the condenser. The liquid in the flask was heated with a free flame until it boiled; and distillation was continued slowly until the water was apparently all over. Water remaining in the condenser was removed by brushing it down into the receiving tube with a brush attached to a copper wire and saturated with liquid. The receiving tube was allowed to cool to room temperature, and drops of water still adhering to the sides of the tube were forced down by a rubber band wrapped around a copper wire. The volume of water was read and the percentage of water present in the drug was calculated by multiplying the volume by two, since a 50 gram sample was used. The procedure was repeated to secure a check.

*Notes on Procedure.* The Bidwell and Sterling distilling tube was not used, because it was found that for practical purposes one could read the volume of water in a Dean and Stark tube to the hundredths of a cubic centimeter just about as well. Sometimes drops of water falling from the condenser did not fall all the way to the bottom of the Bidwell and Sterling tube due to the small diameter of the tube, thus blocking other falling drops of water. This difficulty was overcome by using the Dean and Stark tube which has a much larger diameter. Also, the larger diameter of the Dean and Stark tube made for easier and quicker cleaning.

Dry sand was found not to be necessary, as no serious bumping occurred. Also the distillation was carried out at a much more rapid rate than that called for in the *United States Pharmacopoeia XII*. It was found that distillation could be allowed to go at a rate such that there was a steady, small stream of liquid returning to the flask from the distilling tube receiver; and, at the same time, the vapors of the liquid were condensing in the lower part of the condenser.

It was found that more than 75 cc. of the liquid was required, and in most cases, from 125 cc. to 175 cc. of liquid was used. However with bulky drugs like peppermint and spearmint leaves, from 200 cc. to 300 cc. of liquid was required.

#### INTERPRETATION OF RESULTS

*Time.* In running the moisture determination it was decided to take a reading of the volume of water in the distilling tube every minute for five minutes, taking the first reading one minute after the first drop of water fell from the condenser

to the bottom of the receiving tube. A reading was then taken every minute during the next five minutes, and every five minutes thereafter. This was done because some investigators state that practically all the moisture distills over during the first four or five minutes of distillation.

These investigators, however, found that most of the water came over on an average of 9.5 minutes when toluene was used and in 7.5 minutes when xylene was used, as shown in Table I. The table also shows that xylene removes "most"<sup>2</sup> of the water in a shorter time than does toluene.

The determinations were made with toluene and xylene under as nearly the same conditions as possible. The same weight of drug and the same volume of liquid were used, and the rate of distillation was kept the same. The removal of water was considered to be complete when no change in the volume of water in the distilling tube was noted, after a five-minute period. Distillation with toluene required an average of 37.5 minutes to remove enough water so that the volume as indicated by the calibrated distilling tube showed no increase during the 5 minute period; distillation with xylene required an average of 31.5 minutes. This, too, shows that xylene removes the water in a shorter time than does toluene.

It is evident that the finely powdered drugs gave up their water more quickly than did the more coarsely ground drugs.

Experiments were carried out to ascertain whether a given volume of water could be recovered quantitatively when distilled with separate equal volumes of toluene and xylene; and also to determine which liquid carried over more water in a given period of time.

Table II shows the results of these experiments.

As may be seen in the table, xylene carries over more water in a given time than does toluene. However, it also shows that toluene carries over as much water as xylene when subjected to distillation for a sufficiently long period of time.

*Per cent Water.* In the average time of 37.7 minutes, toluene removed an average of 3.65 cc. of water, while xylene required 31.5 minutes to remove 4.29 cc. of water from the drug; or an average of 7.29 per cent and 8.57 per cent, respectively. It is seen, therefore, that xylene carries over more water in a shorter period of time than does toluene. The accompanying graph (Fig. 3) serves to stress this point.

It would seem from an inspection of the graph that if the distillation with toluene was continued, more water might be made to come over. This was done with a number of drugs and the results are shown in Table III. Unless otherwise stated 50 gm. of drug were used in each case.

An inspection of Table III shows that an average of well over an hour of additional distillation with toluene is required to give a volume of water approximating that obtained with xylene.

<sup>2</sup> "Most" was taken to mean not less than two-thirds of the final volume of water obtained in each determination.

TABLE I  
*Results Obtained from Distillation of Vegetable Drugs with Toluene and Xylene*

VEGETABLE DRUG	VOLUME OF WATER		PER CENT WATER		TIME		VOLUME WATER IN	
	Toluene	Xylene	Toluene	Xylene	Toluene	Xylene	Toluene	Xylene
			cc	cc	per cent	per cent	minutes	minutes
Aconite	3.25	3.95	6.5	7.9	35	32.5	10	10
Agar (25 gm)	3.85	4.4	15.4	17.6	45	40	6	3
Aloe	2.6	3.0	5.2	6.0	40	20	9	3
Althea	3.3	4.25	6.6	8.5	22.5	27.5	10	10
Anise	2.2	2.85	4.4	5.7	35	30	9	5
Aspidium	2.3	3.8	4.6	7.6	105	65	35	20
Belladonna Leaf	2.9	3.4	5.8	6.8	35	35	6	6
Cascara Sagrada	4.5	4.7	9.0	9.4	40	30	8	5
Chondrus	4.1	10 (decomp.)	8.2		55		11	
Cinchona	3.9	4.5	7.8	9.0	30	30	10	7
Cinnamon	4.5	4.8	9.0	9.6	40	35	10	8
Clove	1.7	3.25 (decomp.)	3.4		40		10	
Colchicum Corm	4.5	4.8	9.0	9.6	30	20	10	8
Nux Vomica	4.45	4.9	8.9	9.8	45	25	10	8
Orris Root	4.55	5.1	9.1	10.2	37.5	27.5	10	5
Peppermint	4.1	4.8	8.2	9.6	30	25	8	5
Pomegranate	3.5	4.2	7.0	8.4	20	20	5	4
Physostigma	4.3	4.8	8.6	9.6	50	40	13	6
Quassia	3.25	3.5	6.5	7.0	25	25	7	4
Quillaia	3.9	4.2	7.8	8.4	30	30	8	8
Rhubarb	2.8	3.1	5.6	6.2	30	20	10	10
Senna	3.65	4.2	7.3	8.4	40	30	5	4
Spearmint	3.9	4.5	7.8	9.0	35	25	6	4
Squill	5.85	6.8	11.7	13.6	40	30	10	5
Tragacanth	4.5	6.6*	9.0	13.2	70		22	30
Trifolium	3.1	4.3	6.2	8.6	20	30	5	4
Wild Cherry	3.3	3.9	6.6	7.8	30	30	8	5
Colchicum Seed	2.75	3.3	5.5	6.6	25	35	6	10
Digitalis Leaves	3.0	3.5	6.0	7.0	30	30	7	5
Ergot	2.0	2.3	4.0	4.6	30	20	4	3
Eriodictyon	3.0	3.6	6.0	7.2	25	30	8	8
Eucalyptus Leaves	2.4	2.9	4.8	5.5	30	30	4	5
Gelsemium	3.3	3.65	6.6	7.3	20	25	10	5
Gentian	3.075	3.8	7.1	7.6	37	27	28.5	14.5
Ginger	4.55	4.6	9.1	9.2	47	40	14.5	15
Glycyrrhiza	3.65	4.12	7.3	8.2	34	31	9	6
Hyoscyamus	2.7	3.2	5.4	6.4	45	45	4	8
Kino	5.0	6.0	10.0	12.0	65	65	11	9
Myristica	2.5	2.8	5.0	5.6	25	20	4	4
Myrrh	4.5	5.35	9.0	10.7	40	40	10	5

\* Evidence of decomposition

Since it has been shown by experiment that xylene will carry over more water from the drug in a shorter period of time than toluene, investigations were made to determine why this is true.

The vapor pressure of a substance rises with an increase of temperature. If two immiscible liquids are heated together each will exert its own vapor pressure independently of the other; when the sum of the vapor pressures is equal to the atmospheric pressure the mixture will boil. It is evident that the distillation temperature of the mixture will be below that of either pure component.

TABLE II

SUBSTANCE	VOLUME WATER RECOVERED		TIME REQUIRED TO REMOVE WATER	
	Toluene	Xylene	Toluene	Xylene
	cc	cc.	minutes	minutes
Water (5 cc)	5.0	4.9	20	10
Water (5 cc)	4.9	5.0	20	15
Water (5 cc.)	5.0	5.0	20	15

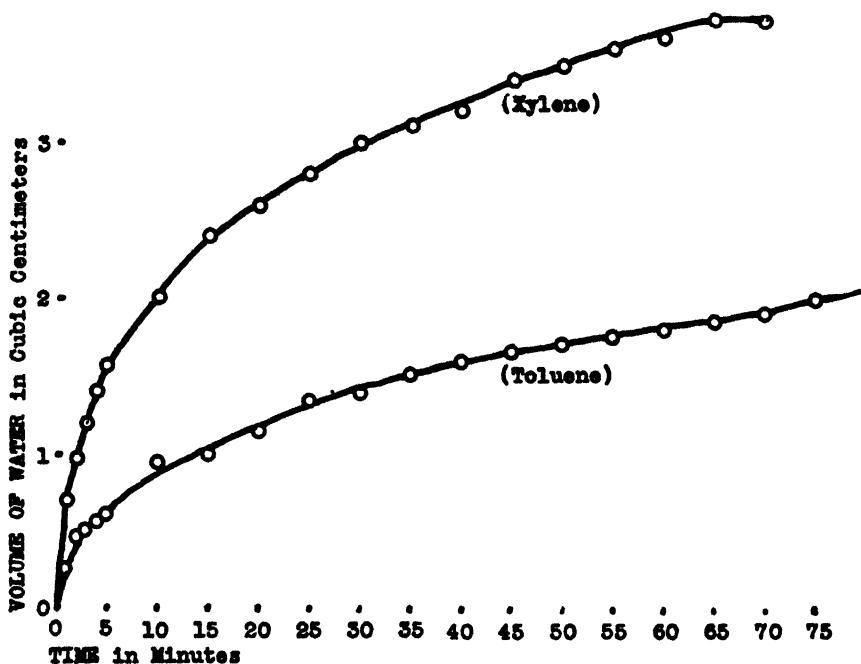


FIG. 3 Curves representing results obtained when Aspidium was distilled with toluene and xylene

Since the vapor pressure of each component is proportional to the number of its molecules in the vapor, we may say,

$$\frac{\text{v.p. } A}{\text{v.p. } B} = \frac{nA}{nB}$$

where  $n$  = number of molecules. For instance, a mixture of toluene and water distils at 91.5°C. (760 mm.). At this temperature the vapor pressure of water

is 556.44 mm., that of the toluene is 203.56 mm. (760 - 556.44). Thus in this case,

$$\frac{\text{v.p. Water}}{\text{v.p. Toluene}} = \frac{556.44}{203.56}$$

To obtain the weights of toluene and water in the distillate we multiply these figures by the respective molecular weights (92.1 and 18). Thus

$$\frac{\text{Wt. Water}}{\text{Wt. Toluene}} = \frac{556.44 \times 18}{203.56 \times 92.10} = \frac{0.53}{1}$$

The distillate contains 0.53 times as much water as toluene.

TABLE III

DRUG	VOLUME OF WATER OBTAINED		CONTINUATION OF TOLUENE DISTILLATION	
	Toluene	Xylene	Volume of water obtained	Additional time required
	cc	cc.		
Agar (25 Gm.)	3.85	4.4	4.3	240
Aloe	2.6	3.0	3.0	60
Belladonna Leaf	2.9	3.4	3.0	60
Cinnamon	4.5	4.8	4.5	98
Colchicum Seed	2.75	3.8	3.0	120
Digitalis Leaves	3.0	3.5	3.5	47
Ergot	2.0	2.3	2.3	90
Eucalyptus	2.4	2.9	2.7	87
Hyoscyamus	2.7	3.2	3.05	120
Kino	5.0	6.0	5.55	120
Myristica	2.5	2.8	2.6	60
Myrrh	4.5	5.3	5.2	180
Nux Vomica	4.4	4.9	4.9	90
Peppermint	4.1	4.8	4.4	90
Pomegranate	3.5	4.2	4.2	53
Physostigma	4.3	4.8	4.7	120
Quassia	3.25	3.5	3.3	60
Quillaja	3.9	4.2	4.2	65
Senna	3.65	4.2	4.2	215
Spearmint	3.9	4.5	4.5	120
Wild Cherry	3.3	3.9	3.7	72

A mixture of xylene and water distils at 93.5°C. (760 mm.). At this temperature the vapor pressure of water is 599.66 mm., that of the xylene is 160.34 mm. (760 - 599.66). Thus in this case,

$$\frac{\text{v.p. Water}}{\text{v.p. Xylene}} = \frac{599.66}{160.34}$$

$$\frac{\text{Wt. Water}}{\text{Wt. Xylene}} = \frac{599.66 \times 18}{160.34 \times 106.12} = \frac{0.67}{1}$$

The distillate contains 0.67 times as much water as xylene, while the distillate from the toluene-water mixture contained only 0.53 times as much water as toluene.

This might offer an explanation as to why xylene carries over more water in a shorter period of time than does toluene.

However, one must consider the manner in which the water is held in the vegetable drug. If the water were free, one might have the same conditions in the distillation of drugs with toluene and xylene as in the above experiment.

It is probable that not enough water is liberated from the plant cells at any one time to give a mixture with a boiling point below that of either component. This was determined by distilling a mixture of 50 gm. of a vegetable drug and toluene. The boiling point of the mixture rose steadily until it reached the boiling point of the toluene (110-111°C.). The water came off over a wide range of temperature. The experiment was repeated using xylene (b.p., 137-140°C.), with the same results. This indicates, therefore, that one obtains more water with xylene in a shorter period of time than with toluene due to the higher boiling temperature of the xylene and not to the production of constant boiling mixtures of toluene-water and xylene-water. The fact that the boiling point of the distilling liquid-vegetable drug mixture rapidly approaches the boiling point of the liquid is evidenced by the decomposition of some drugs with xylene and not with toluene.

*Evidence of Decomposition.* There was definite evidence that chondrus and tragacanth decomposed with xylene and not with toluene. During the distillation with xylene, chondrus darkened after 4.2 cc. of water was collected. The decomposition increased as the distillation was continued, the chondrus becoming charred and the volume of water steadily increasing to 10 cc.

During the distillation of tragacanth with xylene, there was a darkening of the drug after 5.2 cc. of water was collected. The decomposition increased as the distillation was continued, the tragacanth becoming charred and the volume of water steadily increasing to 6.5 cc.

When cloves were distilled with xylene, there was no evidence of decomposition as indicated by darkening, as with chondrus and tragacanth, but after 2.4 cc. of water had been collected, the water continued to come over at a rather consistent rate for over an hour, after which time the volume in the tube became constant. Chondrus and tragacanth, however, continued to give up water as decomposition increased, and no constant volume of water was obtained. This is probably due to the nature of these drugs. The only evidence, therefore, of decomposition of cloves with xylene is the fact that most of the water did not come over early in the distillation, as with other drugs investigated.

#### SUMMARY AND CONCLUSIONS

1. A study of the distillation method for the determination of moisture in vegetable drugs using toluene and xylene as distilling liquids has been made.
2. It has been shown that xylene will remove more water in a shorter period of time from all the drugs examined than will toluene.
3. It would appear that this removal of more water in a shorter time is due to the higher boiling temperature of xylene.

4. Xylene, therefore, would appear to be a better liquid to use than would toluene due to its higher boiling temperature. This temperature, however, was shown to decompose chondrus, tragacanth and cloves. This might be held against xylene in selecting a liquid to be used in the determination of moisture by the distillation method. But this fault is outweighed by the much better results obtained with xylene than with toluene, as has already been pointed out.

5. If toluene were used as the distilling liquid, there would be no decomposition of any of the drugs studied, but the determination would have to be run for several hours to give a volume of water equal to that obtained with xylene; and it is not certain that one could always drive over as much water with toluene as with xylene, no matter how long the determination is run.

6. It is recommended, therefore, that xylene be used as the distilling liquid in the determination of moisture in vegetable drugs; and that for such drugs as will decompose with xylene, toluene should be used.

7. About 70 per cent of all vegetable drugs in the *United States Pharmacopoeia* have been examined; also several non-official ones. It would appear, therefore, that sufficient data have been presented to show the desirability of using xylene instead of toluene as the distilling liquid in the determination of moisture in vegetable drugs by the distillation method.

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# THE ENVIRONMENTAL FACTOR IN SEXUAL INVERSION

BY HARRY W. CRANE

*Department of Psychology, University of North Carolina*

The position to be taken in this paper will be diametrically opposed to the generally accepted explanation of sexual inversion. While there was at one time considerable emphasis upon "suggestion" as a cause for homosexuality, even those who emphasized this basis tended, in the main, to posit some predisposing tendency.

Havelock Ellis, probably the most widely recognized authority in the field of the "psychology of sex," was unequivocal in his position that there is an inherent basis in every case of sexual inversion; and he has vigorously opposed the "now antiquated conception of homosexuality as an acquired phenomenon."<sup>\*</sup> While stating that there are suggestion factors, such as the influence of examples of homosexual behavior enhanced by segregation of the sexes in separate schools, seduction by older individuals of the same sex, and disappointment in normal love, he held that these exciting causes can be effective only in cases in which there is "a latent predisposition." He maintained that these three types of exciting causes were the only ones which he "found influential." He accepted the statement of Dr. G. Frank Lydston that this predisposition consisted of "an organic neurotic basis." That Ellis went even further than this, to the extent of regarding the basis as hereditary in character, is indicated by his statement that "any theory of the etiology of homosexuality which leaves out of account the hereditary factor in inversion cannot be admitted."

The principal argument offered by Ellis against the evidence presented by proponents of the acquired character of inversion, in the nature of cases in which the inversion had followed the introduction of the individual to inverted practices by others, was the contention that many individuals under similar influence did not develop inversion. He therefore concluded that there must have been a predisposing factor in the cases in which the inversion did develop.

The weakness of such an argument is that it ignores the fact that the way in which any particular incident in the life of an individual affects that individual is determined not alone by his inherent equipment but by *everything* that has happened to that equipment from the time of conception on up to the moment at which the particular incident occurs. Thus, an act of seduction by one of the same sex upon a person whose training in the direction of heterosexuality has been rigorous and conducted by adults to whom he has been not only attracted, but whom he respects would be quite different from such a seduction on one without such training. A position such as Ellis took fails to consider the fact that there exists in every individual an environmental predisposition as well as

\* All references to Havelock Ellis are to his position as presented in the third revised edition of Vol. II, "Sexual Inversion," in his series, *Studies in the Psychology of Sex*, Philadelphia: Davis, 1920.

an innate predisposition. It is the position of some that the environmental predisposition is *more specific* in relation to the determination of the *form of behavior* that will result following the presentation of any specific situation at any specific time in the life history of the individual than is the innate predisposition. It would appear also that the environmental predisposition grows in subtlety, complexity, and strength, the greater the age of the individual.

It is also in line with this position that an infinite number of environmental factors occurring in the life history of any normally constituted organism, and that might be duplicated in relation to any other such organism, would predispose to closely similar results in behavior when the two organisms were presented with the same situations. Similar behavior has been used rather than identical behavior, because no two organisms, even though normally constituted, can ever be quite identical.

This method of approach to the fact that not all individuals subjected to suggestive factors develop homosexual tendencies, while others do, seems much more realistic than offering the explanation of a predisposing neurotic inheritance in the one case with its absence in the other. It follows also, in line with the position stated, that one would not expect to find that one traumatic, or one highly satisfying, experience of the so-called suggestive type would, in the majority of cases, result in inversion but that the inversion would later occur as the culmination of a group of environmental factors all tending to influence in the same direction.

A brief presentation of history data furnished by an instructor in another university who talked over with the writer his own difficulties of adjustment arising on the basis of his homosexuality indicates rather clearly some of the incidental occurrences that might be part of the life of any biologically normal individual and would tend to result in a homosexual condition. At about the age of six this boy, in an attempt to satisfy a curiosity (one which is usually present in children) as to possible differences in the physical appearance of the sexes, took a little girl of three under a house that was being constructed in their neighborhood and disrobed her. She quite naturally told her mother about the incident and the mother in turn went to the mother of the boy. As a result he was very severely punished for his behavior. With this early incident probably forming the basis of a recognition of the dangers of interest in the opposite sex, there was a decided emphasis in the same direction by the constant teachings of the boy's father. The father was a minister and was always emphasizing the dangers of "wine, women, and song." According to this man's statement, he grew up with a very definite idea that any interest in, or attachment to, members of the opposite sex was sinful and was frowned upon by society. In the discussion he pointed out that he had never had any intimation that a sex interest in those of the same sex was also frowned upon. When he reached the age of ten or twelve he was in the habit of going swimming at the country swimming hole with a group of boys of about his own age. As is the common practice in such situations, the swimmers were nude. On one of these occasions the boy's genital organs accidentally came in contact with the body of one of the other boys and

he found the resulting stimulation pleasant. In his thinking following this occurrence, there was a dwelling upon the pleasures of that chance contact, with consequent development of sex interest in members of the same sex. It was only after this interest became fully developed that he came to a realization of the attitude of people in general toward one with such an interest.

The one case that will be given in some detail presents a constellation of environmental factors that seems to constitute perfectly adequate evidence of a complete basis for the development of a sexual inversion. At the time this man was first seen he was twenty-five years of age. About two years previously he had attempted suicide by taking strychnine tablets. His stated reasons for this attempt were worry over the suicide of an older brother, which had occurred about two years previous; over his father's health; about the fact that his father had always considered him queer; and over his own feeling that he was unable (satisfactorily) to associate "with boys—or girls either." As more data were secured it became clear that his difficulty in association with both sexes was because of his abnormal attraction to those of his own sex. The unusual intensity of his concern over his father's health also seemed to result from an overattraction to, or fixation upon, his father. More evidence upon this point will be presented later.

The family's economic and social status was that found in the average family in which the father is in the professional class. At the time of the conference the man had completed his high school education and had almost finished his work for an A.B. degree. From the statements made at the conferences there stand out as important contributing factors to this sexual inversion a number of experiences starting when the individual was very young.

The boy's mother had always wished for a daughter. Her first four children, of whom this boy was the last, were boys. Each time she had been disappointed. When this boy was born she called him Peggy after herself, and treated him very much as she would have treated a daughter. Until after he was nine years old she had him sleep with her. This practice would likely have continued had it not been for the birth of a daughter at that time.

The mother spent a great deal of time in petting this boy. He frequently dressed up in his mother's clothes, which practice was probably increased by the fact that it seemed to give the mother a good deal of pleasure. Afternoons when the mother left the home for any purpose the boy was taken to spend the time with her cousin, an unmarried woman of about his mother's age. Here again excessive petting was given the patient and he was taught by this woman to manicure her nails.

During these early years then, most of the patient's time was spent in the company of these older women, and behavior of "feminine type" on his part brought expressions of interest and approval from them. He continued occasional dressing in women's clothing at least until the age of twelve. His acceptance of this "feminine rôle" as satisfying is indicated by the fact that he used to like to play house with his second and third brothers and that they called him "mama."

Other incidences of the mother's behavior towards the boy during these earlier years tend to emphasize her attempts to make a girl of him. Thus, she kept his hair in a long bob until he was seven, crying when she came home one Sunday afternoon to find that the eldest brother had cut it. When he was about six, she made him a white suit to wear to a party, putting lace on the sleeves. In connection with this we find evidence of an attempt at revolt on the part of the boy, for he cut the lace off. This suggests that he was already becoming somewhat aware of the attitude of others towards behavior on his part that he enjoyed when he was in his own home.

The oldest brother's attitude toward this boy seems to have been a particularly important influence. He was "whimsical and erratic and would strike me over the head when he passed. He used to tease me and make fun of me. I was afraid of him. He called me Peg." The man recalled one incident in which, while he was dressed in girl's clothes, this older brother seemed to become sexually interested in him and chased him around the room.

About the age of six the boy was circumcized. This experience left a very vivid impression. The man stated that he remembered very definitely his feeling that his father did not treat him fairly about this operation, saying that he sent him to town with a negro janitor to buy candy. The man indicated that he thought this plan was followed in order that he should be out of the way while preparations were being made for the operation that was to be performed at the home. Upon returning home he was taken into a room, chloroformed, and the operation performed. He said that it was very painful. If one followed psychoanalytic thinking in connection with this incident, one might find grounds for a fear of emasculation as a result of the operation and a consequent resentment towards those who forced it upon him and perhaps a still further acceptance at that early time in his life as a fact that he was actually emasculated.

Somewhat later in that same year the influence of this operation was still further strengthened by another experience. The boy served as a patient in a play situation in which he was undressed by two other boys and the procedure of the circumcision operation reenacted by them. This occurred several times. The man remembered that the manipulations involved in this procedure gave definite pleasure.

When he was "six or younger" this boy and the same two who were involved in the play operation "became interested in two little girls." It was not clear from the man's statement whether the girls were undressed by these boys or that they undressed themselves. At any rate, a sex examination followed. Because the girls got their clothing on wrong-side-out, the mothers became suspicious and the whole story came out. It is probable that punishment and scoldings following this incident played an important part in directing sex interest away from the opposite sex.

The second experience probably accentuating the tendency not to go with girls occurred when the patient was somewhat older. For some time he had been going around with a girl in the community who was quite well-to-do, had her own car, and liked the boy, as he said, "because I was good-looking." One

night as he came out from a moving picture with her he met his oldest brother, who at once commenced laughing. This made the boy very self-conscious and ashamed. Not only was this another unpleasant experience associated with his being with girls, but it tended to emphasize the fact that his oldest brother seemed to think it was peculiar that he should want to be with girls.

When the boy was about fourteen he commenced masturbating. He did this as a definite experiment, having become interested in it through the descriptions he had had from other pupils in the school. The man stated that he had never been able to have an orgasm except through masturbation. He had made an attempt, during the period of residence at a university, to have the normal type of intercourse, going with several other students to one of the cheaper hotels with the intention of attempting intercourse with one of the women there. In recounting this trip he said that he did not have enough whiskey in him so he merely stayed down in the lobby while the other boys proceeded with their plans. It would appear that he was afraid to make the attempt and of the ridicule which might follow on the part of the others in the group if they found out that he was unable to complete the act. This interpretation is strengthened by the fact that he returned later by himself to this same hotel on three different occasions. On two of these occasions he stated that the women with whom he attempted relationships were "so ugly" that it distracted him. On the other occasion he stated that the girl was quite attractive and he told her of his difficulty and she seemed sympathetic. He was, however, unable to complete the act.

Since, during at least the first nine years of his life, and probably for a longer period than that, this boy in a family of four men and one woman was regarded as effeminate by all the males; since he was treated by his three brothers as a girl and, at least by one of them, as a person who could not be expected to have an interest in girls; since he was always treated by his mother as a girl; would it not appear logical that any attachment he might develop towards his father would be on the basis of a daughter-father attitude? In turn, such an attachment to the father would lead to a further strengthening of a general tendency for an attraction towards those of the same sex.

The part played by the nature of the boy's attachment to the father is well shown by an incident that occurred while the boy was living in another city. At that time he found it necessary to consult a physician about some physical ailment. His first visit was followed by more frequent later visits than his physical condition required. On one of these visits he found the office of the physician open, but the physician absent. He took advantage of that situation to place a roll of bills amounting to twenty or thirty dollars in the overcoat pocket of the physician. When the man told of this occurrence, he was asked why he did it. He said that when he was in the second and third grades his teacher, who was employing his father in relation to some legal transactions, frequently sent him to his father's office, which was located only a few blocks from the school building, with notes bearing upon the matter with which his father was dealing. At these times his father would take him up and kiss him which he liked very much. When he entered the office of the physician upon his

first visit he noticed the physician's foot, and something about the entire situation brought back memories of his early experiences when he entered his father's office. He said that this physician reminded him of his father and also of his eldest brother, "besides being very nice looking." The man said he had put the money in the physician's pocket because he wanted to do something for him and also because he thought it might make it easier to have his advances met favorably. In spite of being told by the physician never to return, the man still continued to feel a strong attachment toward him.

At another point in the conference the man volunteered that he had no respect for his father because he considered that he was "bookish," but it is quite clear from the suggested symbolism of the physician in relation to the father and also to the older brother of whom the boy was afraid, that there had been a definite attraction to both of these individuals. The fear element in both the father and older brother situations, suggested putting the question of whether the boy had become sexually excited when he had been whipped by his father. He replied no, but immediately and forcefully volunteered, "but I would like to be whipped by Dr. X," who was the physician in question. There is also present then a very definite masochistic element in the man's sexual behavior.

In interpreting this case of homosexuality with other accompanying sexual abnormalities as entirely the result of environmental influences it is felt wise to point out, and interpret in a similar way, other aspects of this total situation, which would be interpreted by Ellis and others of his school as indicating the presence of a predisposing biological factor. The man stated that his mother was neurotic. There is grave doubt of the soundness of interpreting every case of neurotic condition as due to inherent predisposition. Even if an individual case were due to such a predisposition the result of that neurotic condition upon the children in the family might be solely social in character rather than biological. The suicide of the older brother, plus the fact that many people who knew him interpreted that act as a result of his homosexual tendencies, would again be cited by Ellis as clear evidence of the inherited tendency to sexual inversion. It seems much more logical to suggest again that a boy brought up as a girl and therefore developing behavior usually regarded as "feminine" and even dressing in feminine clothes, would serve as a sex stimulant to the older brothers in the family. Further, since this younger child was actually a male but with certain so-called feminine characteristics, the sex interest of the older brother would be expected to be directed toward other males with similar feminine characteristics. The incident already mentioned in this history of the older brother becoming sexually aroused by the boy when he was in feminine clothing and, at that time chasing him in sexual play, supports this interpretation.

# A FACTORIAL ANALYSIS OF THE RÔLE OF SHOCK IN BRIGHTNESS DISCRIMINATION LEARNING

BY ROBERT J. WHERRY

*Department of Psychology, University of North Carolina*

AND

DOROTHY RETHLINGSHAVER

*Woman's College of University of North Carolina*

The exact rôle of shock in both human and animal learning has been the center of much recent discussion. It is the purpose of this paper to investigate this problem by the use of factorial analysis.

In accordance with the original formulation of Thorndike's *law of effect*, shock was introduced into learning problem procedure as an *annoyer* for wrong responses. The resulting annoyance was supposed to accelerate learning through aiding in the *stamping out* of such wrong responses.

Tolman, Hall, and Bretnall (6) questioned this interpretation as it applied to human learning. In place of the law of effect they suggested three new laws: (a) the law of *motivation*, (b) the law of *emphasis*, and (c) the law of *disruption*. Muenzinger (4) slightly later was questioning the traditional law of effect as it applied to animal learning, and his work suggested what might be called the law of *attention compelling*.

The varying effectiveness of the different methods of stimulation during the various quarters of the learning period in the Tolman, Hall and Bretnall study suggested that the three types of effect noted by them might not be equally prominent at all stages of learning. The fact that measurement of success in trials and in errors did not yield exactly the same conclusions in the Muenzinger study strengthened the feeling that attention to various types of behavior and to various stages in the learning process might yield some interesting results. If shock acted in various rôles it was felt that differential scoring might throw some light upon these rôles.

The present study is based upon the results for 65 rats that learned a simple, one-unit, T-maze, brightness-discrimination problem. All rats received food for making the correct choice. One group of 25 (the NS group) received no further adjunctive stimulation. Another group of 25 (the SW group) received a shock when they made a wrong choice, while a third group of 15 (the SR group) were not shocked for wrong responses, but did receive a shock before getting the food when they made a correct choice. The complete trial-by-trial records for these rats was furnished to the authors by Dr. Karl Muenzinger.

The scoring methods used for this study were selected on the basis of an earlier, unpublished study by Wherry. An analysis of thirty measures of learning had shown the presence of four factors running through the various types of measures. Since the same four factors were found in the present study, their description

will be left until later in the paper. The scoring methods selected were chosen so as to represent the four factors.

The nine scoring methods chosen were the following:

- (1) Total errors in the first ten trials. (This included repetitive errors: thus if a rat entered the wrong alley twice before going to the food, he was charged with two errors.)
- (2) Repetitive errors in the first ten trials
- (3) Total errors to 3 consecutive errorless runs
- (4) Total errors to 15 consecutive errorless runs.
- (5) Trials to 15 consecutive errorless runs.
- (6) Errorless runs in 100 trials.
- (7) Ratio of total errors in trials 51-100 compared to those in trials 1-50.
- (8) Number of errorless runs in 100 trials *after* the next to the last error.
- (9) Wiley's *k*, a measure of the quickness of learning, a sort of *IQ*.

TABLE I

*Number of Times in 100 that Differences in Performance Will in Subsequent Experiments Occur in the Direction Indicated at the Top of the Columns*

SCORING METHOD	NS > SW	NS > SR	SR > SW
1. Errors in first 10 trials	94	14	99.2
2. Repetitive errors (10 trials)	29	16	70
3. Errors to 3 consecutive errorless runs	99.6	14	100
4. Errors to 15 consecutive errorless runs	100	99.7	100
5. Trials to 15 consecutive errorless runs	100	100	66
R 6. Errorless runs in 100	100	100	100
7. Ratio LAST HALF/FIRST HALF errors	100	100	40
R 8. Errorless runs in 100 after the next to the last error	100	99.5	96
R 9. Wiley's <i>k</i>	100	99.0	86

*Note:* In each case these numbers indicate that in this proportion of cases the first group of the pair named at the head of the column will have a poorer record, i.e., will make more errors, take more trials, have less perfect runs, etc. The R's to the left of the methods 6, 8, and 9 indicate that scoring on these items was reversed in order to make this uniform interpretation possible

After the 65 rats had been scored with respect to each of the nine measures above, the mean was computed for each group (SR, SW, and NS) and the critical ratios for the differences between these means were determined for each of the scoring methods. The critical ratios were then transmuted into chances in 100 that the differences would turn out in subsequent experiments in favor of the group named first in each pairing. These results are reported in Table I. The table is to be read as follows: the 94, in the first row of the first column under NS > SW, means that in 94 out of 100 subsequent experiments we could expect that the No Signal group would make more errors during the first ten trials than would the Shock Wrong group. Only 100 or 0 would be significant according to the usual methods of determining reliability of differences. However in the table below, 96 and above meet what is called the Fisher 5% criterion and might be acceptable as indications of *probable* significance.

In his original article concerning these same rats Muenzinger (4) used what amounts to the reversal of our method six (R6) and found, as we have, that the NS group was reliably worse than the SR group which was in turn reliably worse than the SW group. It will be noted that, while some of the other scoring methods (namely 4 and R8) bear out this same set of conclusions, the table as a whole does not show agreement. Methods 5, 7, and 9 point to a greater equality, if not reversal (method 7), in the relationship between the SW and SR groups.

TABLE II  
*Intercorrelations, Residual Coefficients, and Factor Loadings*

Intercorrelations

	1	2	3	4	5	R6	7	R8	R9
Residuals	1	.796	.750	.541	.173	.369	-.080	.197	.072
	2	.037	.442	.226	-.014	-.001	-.144	.026	-.152
	3	.000	-.016	.539	.162	.447	-.087	.168	.088
	4	.033	.014	-.011	.772	.912	.366	.577	.512
	5	.016	-.031	-.016	.033	.700	.497	.468	.548
	R 6	-.040	-.010	.007	-.022	-.002	.541	.761	.685
	7	.006	-.011	.023	-.022	-.001	-.003	.736	.672
	R 8	.015	-.001	.017	-.039	.023	.025	-.013	.853
	R 9	-.019	.020	.012	.006	-.041	-.048	.036	-.010

M residual  $\pm \sigma = -0.001 \pm .023$ ; N = 65.

Factor Loadings

	CENTROID				ROTATED				
	I	II	III	IV	PE	RE	LE	SI	MA
1	.61	-.74	-.18	-.08	.86	.46	.08	.09	.97
2	.33	-.69	-.35	.20	.55	.62	-.18	-.02	.72
3	.55	-.56	.14	-.32	.78	.10	.31	.00	.71
4	.90	-.05	.39	.08	.36	.24	.76	.45	.97
5	.68	.24	.29	.31	-.07	.26	.62	.50	.71
R 6	.89	.24	.30	-.18	.31	-.09	.66	.64	.95
7	.54	.59	-.20	.14	-.18	.03	.10	.81	.70
R 8	.78	.44	-.32	-.18	.21	-.08	.07	.93	.92
R 9	.69	.55	-.16	-.17	.05	-.17	.21	.87	.83

Methods 1, 2, and 3 point to a similar reversal of the NS and SR relationship, as well as less certainty of even the NS-SW and SR-SW differentiations.

Since each of these scoring methods could have resulted from the operation of several factors, it seemed advisable to apply factor analysis in an attempt to unravel the contradictory relationships mentioned in the preceding paragraph. The nine scoring methods were intercorrelated (Pearsonian product-moment method) and a Thurstone centroid factor analysis applied. Four factors were extracted and their extraction justified by Tucker's criterion (3). The original correlations, their fourth order residuals, and the centroid and rotated factor loadings are given in Table II.

## NAMING THE FACTORS

The factor labeled PE in Table II had the following significant loadings (above .30):

Total errors on first 10 trials . . . . .	.86
Total errors to 3 consecutive errorless runs	.78
Repetitive errors in first 10 trials	.55
Total errors to 15 consecutive errorless runs	.36
Errorless runs in 100 trials	.31

Since the heaviest loadings occur on the more lax criteria, this factor has been called *preliminary errors*. It might be taken as a measure of the adequacy of early hypotheses (in the Krechevsky sense). The moderate loading on repetitive errors is probably best explained on the basis that animals who made more initial errors had more chance to repeat them. The slight loadings on the more stringent criteria seem logical when it is realized that these preliminary errors have contributed in part to the formation of these scores.

The second factor, labeled RE, had only two significant loadings:

Repetitive errors on first 10 trials	.62
Total errors on first 10 trials	.46

The factor was called simply *repetitive errors*. The relatively high loading on total errors in this first ten trials seems quite logical when it is realized that it is during these first ten trials that most repetitive errors occur. The dropping out of these repetitive errors as learning progresses is shown by the sub-significant loadings of .10 and .24 for this factor on scoring methods three and four, total errors to reach the criterion of 3 and 15 successive errorless runs respectively.

The third factor, labeled LE, had the following significant loadings:

Errors to 15 consecutive errorless runs	.76
Errorless runs in 100 trials (reversed)	.66
Trials to 15 consecutive errorless runs	.62
Errors to 3 consecutive errorless runs	.31

This factor apparently has to do with and has been named for *later errors*. This name seems justified on two grounds: (a) the factor appears most strongly in all of the more stringent criteria, while it is absent or low on all of the criteria based on early performance; (b) and it is most marked in criteria based upon errors. The relatively high loading on the most stringent of the trial criteria seems best explained by the fact that an animal who continued to make even an occasional error late in the training period would not be able to satisfy these stringent criteria. This factor might be thought of as being related to the ability of the animals to profit by their mistakes, i.e., to reflect the "stamping out of wrong responses." After discussing the next factor, we shall consider further this interpretation of the present factor.

The fourth and last factor, labeled SI, had the following significant loadings:

Errorless runs in 100 trials after the next to last error (reversed scoring)	.93
Wiley's $k$ (reversed scoring)	.87
Ratio of last half to first half errors	.81
Errorless runs in 100 trials (reversed scoring)	.64
Trials to 15 consecutive errorless runs	.50
Errors to 15 consecutive errorless runs	.45

This factor has been named *slowness of insight* or *slowness in comprehending or attending to the main relationship involved in the problem*. This interpretation is based upon the following observations:

- (a) The highest loading, .93, is found on a scoring method which approximates one common interpretation of the term *insight*, namely the sudden appearance of the correct response followed by invariably correct performance from then on. In our scoring method we did allow one error. The sooner this insight occurred the longer this unbroken chain of successes at the latter end of the 100 trials would be.
- (b) The second highest loading, .87, is on Wiley's  $k$ , which has been shown to be a measure of the sharpness in the bending of the learning curve, a measure of speed of learning. It has been assumed by the Wileys to be a measure of the intelligence of the animal.
- (c) The absence of the factor on straight error criteria except the most stringent, and the slightly higher loading on trial as compared to error criteria even when both were equally stringent

If the last factor can be taken as a measure of the animal's ability to profit by its mistakes, this factor seems to reflect the animal's ability to profit by its correct responses, i.e., to reflect the "stamping in of correct response."

The finding of these two factors representing two phases or aspects of performance late in the learning situation seems to be a reflection of the double aspect of the law of effect as originally formulated by Thorndike. It also calls to mind the rational learning curve developed by Gulliksen (2) on the basis of the law of effect, and suggests the usefulness of his more complex learning curve as contrasted to the simpler one of Thurstone (5) recently put into usable form by the Wileys (7). It further research shows the validity of distinguishing between these two aspects of learning, it is to be hoped that someone will make possible the finding of the constants  $c$  and  $k$  in Gulliksen's equation by some such simple process as that derived by the Wileys for the single  $k$  of the Thurstone formula, which we used as scoring method 9.

#### PREDICTING THE FACTOR SCORES

The next step was to secure the regression equations so that each of the rats might be scored for each of the four factors. These equations were secured by using the factor loadings as criterion correlations and computing multiple regression equations involving those tests which were high on each given factor and relatively low on the remaining factors. This selection of tests was made so as to keep at a minimum the intercorrelation between the various factor scores. The four regression equations, in gross score form and with all tests indicating

original scoring (the reversals necessitated in the factor analysis have been taken care of in the signs) were:

$$\begin{aligned}
 PE &= 5.86X_1 - 2.83X_2 + .43X_3 + 7.24 \\
 RE &= -2.05X_1 + 7.22X_2 + .53X_4 + 44.80 \\
 LE &= .88X_4 + .19X_5 - 1.01X_6 + 113.82X_7 + 96.79 \\
 SI &= .05X_5 + .50X_6 + 24.44X_7 - .34X_8 - 72.84X_9 + 22.53
 \end{aligned}$$

As suggested above an attempt was made to avoid intercorrelation between our factor scores in so far as possible, but, with overlap of the factor present to some extent in all of the variables, complete success was impossible. The intercor-

TABLE III  
*Validity and Intercorrelations of the Factor Scores*

Factor	INTERCORRELATIONS				VALIDITY
	PE	RE	LE	SI	
PE		.322	.493	.080	.906
RE			.340	.191	.650
LE				.246	.819
SI					.966

TABLE IV  
*Mean T-Scores and the Number of Times in 100 that Future Experiments Will Show Differences In Performance in the Direction Indicated at the Heads of the Columns*

Factor	MEAN T-SCORES			PROBABLE PERCENTAGE OF SUPERIORITY ON REPEATED EXPERIMENTS		
	NS	SW	SR	NS > SW	NS > SR	SR > SW
PE	51.5	43.7	56.3	100	1	99
RE	51.5	49.3	50.5	64	57	77
LE	57.8	42.5	50.7	100	99.2	100
SI	57.9	43.9	47.0	100	99.5	76

*Note:* This table should be interpreted in the same manner as Table I.

relations of the factor scores as computed by the regression equations given above, along with the multiple correlation coefficient (factor loading for the batteries) is given for each factor in Table III.

The final computations consisted of securing the means, sigmas of means, critical ratios, and probable percentage of superiority on repeated experiments for each of the groups of animals on each of the four factors. The means and the number of times in 100 that in future experiments the results will turn out as indicated at the head of each column is given for each of the factors in Table IV.

Results for the first factor, PE-Preliminary Errors, show clearcut differences between the three differently motivated groups. The Shock Wrong group made fewest preliminary errors ( $M = 43.7$ ), the No Shock group was second best ( $M = 51.5$ ), and the Shock Right group made the most early errors ( $M = 56.3$ ).

As good an explanation as any for these results seems to be that the groups which were shocked tended successfully to avoid the shock, or, as with the Shock Right group, to delay its reception during the initial stages of the problem. Since the shock as such was not in evidence until after choice, this must mean that the two shocked groups had learned to pay attention to the crucial stimulus (the dark-light relationship) even during these early trials. Since avoidance of this shock meant a correct choice for the Shock Wrong group, they made the fewest errors. For the Shock Right group, however, any ability to avoid the alley containing the shock meant the making of an error, and this group made more errors than either of the others during this early period. It should be noted of course, that these responses were *wrong* only in the sense that they did not lead to food (the problem set by the experimenter) but were actually *correct* if we assume that the animals were attempting to avoid the shock, or to delay the experience of taking it, since they did finally have to enter the correct (shock) alley to terminate a run. The above interpretation would seem to be an indication of the action of the Tolman-Hall-Bretnall law of motivation and to agree with Thorndike's original statement of the law of effect. The authors would like to stress again that, while from the experimenter's viewpoint the Shock Right group was reliably worse than the Shock Wrong group, they were about equally *successful* in their attempts to avoid the shock. Apparently, even at this early stage, these two groups were already paying attention to the light-dark difference. This last statement seems to agree with the Muenzinger law of attention compelling. Evidence for this law is most striking, however, in relation to the SI factor which we will discuss later.

With respect to the second factor, RE-Repetitive Errors, the three groups are seen to be only slightly and unreliably differentiated. This would seem to indicate that the Tolman-Hall-Bretnall law of disruption, which they found for humans, cannot be verified for rats on the basis of this experiment. This conclusion is based upon the twin assumptions that disruption implies emotionality and that repetitive errors are evidence of such emotional disturbance, the latter assumption being taken from Everal (1). Neither the factor scores nor the original scoring method of repetitive errors in the first ten trials show any reliable differences.

Turning to the third factor, LE-Later Errors, we find the three groups again reliably separated. The No Shock group makes the most errors, the Shock Right group is second, and the Shock Wrong group makes the fewest errors. One possible explanation of these results is as follows: The Shock Wrong group is superior for two reasons: (1) they have caught on, know what the problem is about (see next factor), and (2) they have been punished for wrong responses, and therefore tend always to use the knowledge implied in the first statement, i.e., they have an added motivation beyond that of receiving food. At the other extreme is the No Shock group, which is inferior for the same two reasons in reverse: (1) this group is very slow in catching on to the correct response (see next factor), and (2) they have had no particular punishment for wrong responses except a slight delay in reaching food and therefore are more apt occasionally to

repeat such responses. The Shock Right group, which was intermediate with respect to these later errors, has these two reasons in opposition to one another: (1) like the Shock Wrong group they have been successful in getting insight into the problem, as is shown by the next factor, *but* (2) like the No Shock group they have not had particular punishment for wrong responses; indeed they have been actually punished for making correct responses, thus bringing the motive to secure food in actual opposition to the motive to escape shock.

That this opposition in motivation has not left the Shock Right group actually inferior to the No Shock group is probably best explained by some theory of adaptation to shock on the part of this Shock Right group. In the early part of the experiment the shock was a stronger motive than was the food; this seems indicated on the grounds that the Shock Right group made even more *Early Errors* than did the No Shock group. In the later stages, as the animals become accustomed (adapted) to the shock, however, the motive to avoid shock becomes relatively weaker than the motive to get the food immediately, and so becomes inoperative except in occasional and sporadic instances. It is these sporadic instances on the other hand which explain the relatively poorer performance of the Shock Right as compared to the Shock Wrong group.

With respect to the last factor, SI-Slowness of Insight, we find that the Shock Right and Shock Wrong groups are unreliably differentiated although both of these groups are reliably faster in catching on than are the No Shock animals. It will be recalled that even in the early trials these two successful groups had been able to respond effectively to the light-dark relationship, although at that time they seemed to be using it as a cue to avoid shock rather than to secure food. Their attention had, however, been centered upon this cue and it was therefore easier for these groups to make the discovery that this light-dark relationship was also the cue to the location of the food. This factor seems then to give support to the Muenzinger law of attention compelling.

#### CONCLUSIONS

1. This study supports the Muenzinger conclusion that shock tends to cause earlier and more constant *attention* upon vital cues. The two groups having this added incentive toward attention made the discrimination sooner, i.e., made reliably lower scores on the factor of Slowness of Insight.

2. If it be assumed that shock motive, in contrast to the food motive, decreases in psychological intensity with repeated applications, then there is no evidence to dispute the traditional statement of the law of effect.

a. In the early trials there was a tendency for both shocked groups to avoid the shock, the Shock Wrong group making fewer errors than the No Shock group while the Shock Right group made more errors than the No Shock group.

b. In later trials the Shock Wrong group continued to avoid the shock (and/or to go to the food) and continued to have a lower error score than either of the other groups. The Shock Right group, forced to take shock on every trial, however, had the motive to secure food in actual opposition with

motive to avoid shock. The motivation to get food seemed to increase relatively to the motivation to avoid shock, possibly through adaptation to the latter, and this group finally came to use their earlier mastery of the required discrimination so effectively that although they remained inferior to the Shock Wrong group, they at least made fewer late errors than did the No Shock group.

3. If we assume that disruption should have shown up in connection with repetitive errors during early trials, then there was no evidence for this effect in these rats.

4. The utility of *multiple scoring* in discrimination learning and the applicability of factor analysis to the clarification of this complex problem has been demonstrated.

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# STUDIES OF RESPONSE GENERALIZATION IN CONDITIONING.

## III: STIMULUS VARIATION AS A FACTOR IN RESPONSE GENERALIZATION

BY DELOS D. WICKENS

*The University of Wisconsin*<sup>1</sup>

### INTRODUCTION

The conditioned response, according to Pavlov (4), was the attachment of a specific response, or reflex, as he called it, to a new stimulus. A certain reflex is isolated out of the subject's behavior repertoire by the action of the unconditioned stimulus, and, through a process of pairing, this isolated reflex becomes attached to a new stimulus. Conditioning, then, consists of connecting a new stimulus to an isolated response. The Pavlovian laboratory produced research which pointed out that the new stimulus was not specific in nature and the response could be evoked by other similar stimuli, even though these had not been associated with the unconditioned stimulus. This was called irradiation by Pavlov; later American investigators have named it stimulus generalization (3).

Although considerable interest was shown in the fact of the non-specificity of the conditioned stimulus, a similar interest in the variations of the response was not evidenced. Probably as a result of the fact that the salivary response was the usual unconditioned response, variations in the quantitative rather than the qualitative aspects of the behavior was of major concern to investigators. The results of these researches led to the view, usually implicit, that the conditioned response was a qualitative stereotype of the unconditioned response, and that it could be expected to vary in magnitude but not in kind.

Later work, particularly by American investigators, began to indicate that this concept of the conditioned response was inadequate (2). Evidence began to accumulate which showed that considerable variation in the qualitative aspects of the conditioned response were to be expected, and that it was not unusual for the conditioned response to differ considerably from the unconditioned response in form as well as in magnitude.

In several studies by the present writer (5, 6, 7), the question of how the conditioned response is affected by perceptual factors at the time of its elicitation was raised. In these studies an extensor movement of the finger was conditioned, and then the arm was turned to a position so that the opposite or flexor movement would have to be made if the finger was to be removed from the electrode.

The electrode itself was placed upon a floating platform, so that either an extensor or a flexor movement could be recorded. The conditioned stimulus was then sounded, without, of course, there being any presentations of the unconditioned stimulus in this new position. In all of these studies the general trend of the results was the same. In all instances the majority of the subjects responded by

<sup>1</sup> On leave from University of Wisconsin to National Defense Research Project. See p. 360.

making *flexor* rather than the originally conditioned extensor movements; a few subjects failed to respond at all, and a very small number of subjects responded by making extensor movements.

At the termination of the experimental session the subjects were questioned concerning their thoughts at the time the hand was turned over and the conditioned stimulus was sounded. Explanations of behavior were not readily forthcoming, and most of the subjects simply said that their fingers acted in the way they did more or less of their own accord; there was no satisfactory way of discriminating between the subjects who made extensor movements and those who made flexor movements on the basis of verbal reports.

Thus, under presumably identical conditions, two opposing types of response occurred. For the majority of the subjects the conditioned response which did occur was modified by the new environmental situation. The response had become generalized and was non-specific in nature. In a few instances the original form of the response persisted even though this form would have nonadaptive value in the new situation.

The occurrence of these contradictory responses in the same experimental situation indicated the need for further research which would be directed toward analyzing out the factors of which response generalization in conditioning is a function. The present study is an attempt to investigate the interaction of stimulus generalization and response generalization. Specifically, is the generalized response more or less likely to occur if a tonal stimulus in the test situation is the same as or different from the stimulus used in the training situation?

#### PROBLEM

Is response generalization affected by the nature of the conditioned stimulus<sup>8</sup> used?

#### SUBJECTS

The subjects in this experiment were 58 volunteers from the elementary psychology class at the University of Wisconsin.

#### APPARATUS

The conditioning board consisted of a plank about three feet by one foot. This was fixed with adjustable restraining devices so that the subject's hand and arm could be fastened securely in position. Straps were placed over the subject's forearm, wrist, and the back of his hand. A block at the back of the elbow prevented the subject from making horizontal withdrawal movements.

One of the shock electrodes was strapped to the subject's wrist, while the subject's middle finger rested on the other electrode. The shock circuit could be broken by removing the finger from the electrode. The tone, used as the conditioned stimulus, was delivered through earphones worn by the subject.

Finger responses made by the subject were recorded by means of electrical contacts. A narrow strip of metal, hinged at one end, rested on the top of the subject's finger. When the finger was lifted off the electrode, this metal strip

came in contact with a metal spring above it, thus closing the circuit. Downward movements of the finger could also be recorded. The electrode on which the finger rested was attached to a platform which was supported by a spring. The platform was pierced by a bolt. When the platform was depressed, the bolt dipped into a mercury well, and the recording circuit was closed. Both the upward and downward response circuits could be adjusted so that movements of the finger no greater than  $\frac{1}{16}$  of an inch could be recorded. Lights on a control panel in the experimenter's room indicated whether an upward or a downward response occurred.

The shock stimulus was delivered by a high voltage power pack. This, coupled with the facts that one electrode was strapped on the subject and that the other was small in area, served to maintain the shock at a fairly constant value from stimulation to stimulation.

The tone was delivered to the subject by means of a Maico Audiometer.

The time relations between the conditioned and the unconditioned stimuli were controlled by a timing disk. This was a disk driven by a constant speed motor. On it were lugs which moved into contact with brushes, and, when contact was made, relays were closed and the stimuli were applied. The conditioned stimulus occurred for .5 of a second, and was followed, .4 of a second after its onset, by the unconditioned stimulus. The unconditioned stimulus had a duration of about .1 of a second. At a constant interval of time before the occurrence of the conditioned stimulus, a clock which measured time in hundredths of a second was started. This clock was stopped by the subject's response. In this manner latent times in hundredths of a second could be recorded.

The subject and experimenter were seated in adjacent, sound proofed rooms. A one-way mirror between subject and experimenter permitted the experimenter to observe the subject without himself being observed.

Switches on a control panel made it possible to administer the conditioned and the unconditioned stimulus independently of each other, as well as permitting the variation of the inter-stimulus period.

#### PROCEDURE

In general, the procedure of the experiment consisted of conditioning the subjects while the hand was strapped palm down, so that the extensor movement removed the finger from the electrode. Following this, the hand was turned over, palm up, so that a flexor movement was required to lift the finger from the electrode. While in this new position, the conditioned stimulus *alone* was sounded 10 times. For 27 of the subjects the same tone was employed in the transfer test situation as was employed in the training situation. The tone was 50 decibels above the threshold with a frequency of 256, 512, or 1024. For 32 of the subjects the test tone differed from the training tone by either one or two octaves. The same frequencies and intensities were used for this group as for the same tone group.

After the subjects came into the experimental room, the experimenter explained to them that they were to serve in a conditioning experiment. They were then

told that conditioning would be accomplished by pairing a shock with a tone, and that, after a while, they would develop a tendency to respond to the tone alone. They were asked neither actively to try to respond to the tone, nor to inhibit the response if they felt like making it. They were told that the experiment would begin with a test of threshold to two tones, followed by a test to determine what level of shock was to be used.

Rough threshold measurements for two tones were made by the simple clinical method of lowering the intensity of the tone until the subject reported that he could no longer hear it, and then increasing the intensity until the subject reported that it could be heard. Since intensity was controlled in steps of five decibels, there was usually little difference in the up and down threshold. The intensity level of the tone that was used in the experiment was 50 decibels above the threshold.

Shock intensity varied from subject to subject. It was determined by stepping up the level of the shock until the subject reported that it was as strong as he desired to take. Occasionally, if the latent time of the shock reaction was long, the experimenter increased the shock during the experiment.

Tests to the conditioned stimulus followed the adjustment of the shock. Three stimulations of the tone alone were given. The particular tonal frequency employed was of the same value as the frequency and intensity that was later used when the hand was turned over. Only one subject reacted to this tone by lifting the finger at this time, and this subject was not employed in experiment.

Following these tests, the unconditioned stimulus alone was presented three times. The latent times were recorded, and the shock was stepped up slightly if they were particularly long.

Immediately after the last test to the unconditioned stimulus, the training series was begun. This consisted of 130 paired stimulations of tone and shock. The time intervals between paired stimulations varied from 5 to 35 seconds in a prearranged regular plan. The latent times of the responses were recorded after each reaction.

After the last paired stimulation was given, the experimenter went into the subject's room and stated, "Now I want to turn your hand over so that the same finger is on the electrode, but your palm is up." If the subject asked any questions, the experimenter replied that he would discuss the entire experiment with him later. The hand was then turned over and the recording contacts adjusted.

The transfer test procedure consisted of 10 stimulations of the tone *alone*. For 27 subjects a tone the same as that of the training situation was used. For 16 subjects a tone differing by one octave from the training tone was used. For 16 subjects a tone differing by two octaves was used. In all cases the intensity level was 50 decibels above the subject's threshold. The time intervals between stimulations varied in prearranged random order from 5 to 35 seconds.

#### RESULTS

1. *General results.* The results of the study are presented in Table I. Column 1 identifies the groups and column 2 gives the number of subjects in each group. The next 3 columns indicate how the subjects reacted when the hand was turned

over. Column 3 shows the number of subjects who failed to respond at all; column 4 shows the number who gave flexor or generalized responses; and column 5 lists the number of subjects who continued to make extensor or specific responses.

2. *Comparison of the frequency of generalized and specific responses in the two groups.* The data of greatest interest in the present study are those which present a comparison of the tendency to make specific or generalized responses in the two groups. These comparisons are available from the material presented in Table I. Twenty-five of the subjects who were tested to the same tone responded. Of this group, four gave specific responses—that is, made a response that in muscular terms was the same that they had been making during the original training—and 21 subjects reacted with a generalized response—that is, changed the form of the response from an extensor movement to a flexor movement. In the group which was tested to a different tone, 22 subjects responded in the transfer situation. Everyone of these subjects made the generalized response.

TABLE I

*Showing the number of subjects who failed to respond, or gave specific or generalized responses when the hand was turned over*

GROUP	NUMBER	NO RESPONSE	GENERALIZED RESPONSE	SPECIFIC RESPONSE
Generalized tone	32	10	22	0
Same tone	27	2	21	4

A comparison of the two groups by the method of Chi Squared produces a Chi Squared value of 3.8477. Such a Chi Squared value as this would occur by chance just a little less than 5 times in 100. One may state with a moderate degree of confidence that the difference in behavior of the two groups was not due to chance variation.

#### DISCUSSION

The statistical analysis presented in the results indicated some tendency for the group which was tested to the generalized tone in the transfer situation to be more likely to give a generalized response than the group tested to the same tone.

In other words, it would appear that response generalization may be a function of stimulus generalization, and that the introduction of new perceptual factors in the situation is not as likely to result in a modification of the response if the stimulus remains the same as it is if the stimulus is somewhat modified. It suggests that the organism learns to make a certain response to a certain stimulus and the response persists in the presence of this stimulus, even though it is apparently nonadaptive, and even though another kind of response would seem to be more appropriate.

While the results would not be contrary to any learning theory, they are especially pertinent to the Guthrie type of theory which places considerable

emphasis on the prediction of the muscular form of the response, and on the factors which tend to produce variation in its form (1).

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# THE EXCYSTMENT PROCESS IN THE CILIATE DIDINIUM NASUTUM<sup>1</sup>

BY C. DALE BEERS

*Department of Zoology, University of North Carolina*

## PLATES 9-11

It is well known that nearly all freshwater ciliates have the ability to produce resting or protective cysts, usually in response to unfavorable environmental conditions, and that they may remain viable for months or even years in this quiescent state, ultimately to re-emerge when conditions are presumably more favorable. Although the literature on the various aspects of cystment is fairly extensive, the factors which induce such emergence and the mechanism by which the ciliate escapes from its tough, enveloping membranes are still inadequately understood.

The evidence adduced from the studies of Weyer (1930), Barker and Taylor (1933), Haagen-Smit and Thimann (1938) and others indicates that the process of excystment is initiated by the presence of various organic substances in the environment, but there is evidence from results obtained with distilled water that changes in the osmotic properties of the medium may sometimes suffice to induce excystment (Goodey, 1913; Garnjobst, 1928; Johnson and Evans, 1940; Beers, 1945).

As to the mechanism of rupture of the membranes, it is generally agreed that osmotic changes are of primary importance, as Fabre-Domergue (1888) and Bütschli (1889) pointed out. The cytoplasm imbibes water during the early stages of excystment and increases appreciably in volume, though much of the water accumulates in one or more discrete vacuoles in the cytoplasm. Thus, in *Vorticella nebulifera* (Fabre-Domergue), *V. microstoma* (von Brand, 1923), *Pseudomicrothorax agilis* (Penard, 1922), *Stylothenes sterckii* and *Euplates taylori* (Garnjobst, 1937) there is only a single, relatively enormous vacuole, which appears to be identical with the contractile vacuole; whereas in *Trachelium ovum* (Fabre-Domergue) and *Tillina magna* (Beers, 1945) there are many sizable vacuoles. The gradual increase in volume exerts a progressively mounting pressure from within against the cyst membranes, which, having only moderate elasticity, are forcibly ruptured.

The studies of Goodey on excystment in *Colpoda cucullus* directed attention to a second factor which participates in the excystment process, viz., the elaboration by the organism of an excystment enzyme. In *Colpoda*, according to Goodey, there are only two cyst membranes—ectocyst and endocyst. Through the imbibition of water the ectocyst is ruptured, and the ciliate, by now fully differentiated, escapes from its outermost membrane, though it is still im-

<sup>1</sup> Supported in part by a grant from the Smith Research Fund. I am indebted to Dr. D. P. Costello for valuable advice and assistance in making the accompanying photomicrographs.

prisoned in the thin, transparent, spherical endocyst. Gradually the endocyst increases in diameter, its wall becomes thinner, and finally it disappears as if dissolved in the medium. Thus the colpoda is set free. Upon subjecting the endocyst to a variety of chemical tests, Goodey found that it is digested completely by diastase, but not by pepsin or trypsin. He concluded that it is of a carbohydrate nature and that the colpoda "liberates itself by the rapid digestion of the endocyst by means of a powerful enzyme which it secretes." For the carbohydrate of the endocyst he proposed the name "cystose," and for the enzyme, "cystase." The observations of Johnson and Evans (1940) and of Beers (1945) on the excystment of *Woodruffia metabolica* and *Tillina magna*, respectively, are in general accord with those of Goodey, in that the ciliate, upon escape from its outer membranes (of which there are two—ectocyst and mesocyst) is still enclosed in an endocyst. Soon the endocyst increases in diameter and disappears rather quickly in the medium, thus freeing the ciliate. In *Styloynchia mytilus* the endocyst is likewise dissolved during excystment, and the presence of an enzyme is evident, according to von Brand. A point which has not been made clear in the foregoing discussion concerns the means by which a ciliate, still in its endocyst, manages to escape from its outer investments. The method is variable. Often it is not adequately explained, probably because of difficulties of observation. In *Tillina* the elastic recoil of the ectocyst and mesocyst, following their simultaneous rupture when their elastic limits are exceeded, is so pronounced that the ciliate and its enveloping, turgid endocyst are somewhat forcibly extruded into the medium (Beers, 1945). In *Didinium*, as will be seen, the rupture of the outer membranes is less violent, and a large excystment vacuole aids in expelling the ciliate.

As a matter of fact, there seem to be surprisingly few complete accounts of the excystment process in ciliates, though incidental observations are plentiful. Apparently most investigators have been interested primarily in other aspects of cystment, such as the nuclear phenomena (Ivanić, 1928, 1934; Beers, 1935; Kidder and Claff, 1938; Taylor and Garnjobst, 1941; Evans, 1944), the rôle of external factors (Stolte, 1924; Beers, 1927; Michelson, 1928; Taylor and Strickland, 1935; Johnson and Evans, 1941), the properties of the membranes (Ilowsky, 1926), and resistance to adverse environments (Bodine, 1923; Taylor and Strickland, 1936). So far as I know, there exists no satisfactory account whatsoever of the excystment process in the remarkable predacious holotrich *Didinium nasutum*. The present paper furnishes such an account, with observations based exclusively on living, rather than fixed, specimens and supplies further data on the factors that induce excystment.

#### METHOD OF INDUCING ENCYSTMENT

Resting cysts were obtained by the completion-culture method. First, a pure line was established with an excysted specimen. This line was maintained in isolation culture at 22°C., spring water serving as the fluid medium and *Paramecium multimicronucleatum* (grown in timothy infusion on wild bacteria) as the food. (For details of the cultural needs of *Didinium* reference may be made to

Beers, 1937.) With progeny of the pure line small encystment cultures were prepared as follows: Two didinia were removed to 0.2 cc. spring water contained in the depression of a culture slide. Approximately 500 paramecia, which had been washed and concentrated centrifugally in spring water, were added, and the slide was stored in a moist chamber. At 22°C. this number of paramecia was usually entirely consumed by the original two didinia and their progeny within 36 hours, with the production of approximately 128 didinia (representing six generations). Of these, 75-100 encysted in the following twelve-hour period, whereas the others remained active until they perished of starvation. The failure of some didinia to encyst in the absence of food has been reported by several investigators (Mast, 1917; Beers, 1927; Reukauf, 1930; Rammelmeyer, 1931), but it has not been satisfactorily explained.

These cysts were used in excystment studies with uniform results, when they were anywhere from ten days to three months old. Spontaneous excystment has never been reported in Didinium, and such cysts, if suitably protected from evaporation of the medium, remain viable for many years (Beers, 1937a). In practice, twenty or more of these small encystment cultures were prepared at one time, rather than a few large cultures, since the conditions of growth and encystment can be controlled and standardized more satisfactorily in small cultures. Although encystment in these cultures never occurred until the food supply was exhausted, it may not be asserted that encystment was the result solely of absence of food, since the metabolic waste products of the paramecia and didinia were present in the cultures, and bacteria as well. Nevertheless, the procedure affords a reliable and reproducible means of obtaining an abundance of normal cysts, in agreement with the observation of Mast and Ibara (1923) that "rapid and extensive reproduction of didinia confined to a small space seems to favor excystment."

#### METHOD OF INDUCING EXCYSTMENT, WITH CONSIDERATION OF SOME EXCYSTMENT-INDUCING FACTORS

In order to induce excystment in Didinium it is the usual practice to replace the old encystment medium with hay infusion from a flourishing Paramecium culture (Mast, 1917; Beers, 1930, 1935). In my experience, and in agreement with Mast's findings, this method yields a somewhat low percentage of excystment, and the time required amounts to 24 hours or more. In the present study a more effective procedure was employed, though the method is in need of further improvement and the medium, because of certain variables, cannot be looked on as infallible. To prepare this excystment medium, 20 cc. of a 0.5 per cent. aqueous solution of peptone ("Bacto-Peptone," Difco) was inoculated with bacteria from the encystment cultures and incubated 18 hours at 28°C. The abundant flocculent masses of bacteria, which consisted chiefly of filamentous forms, were removed by filtration through Whatman No. 43 paper, and the filtrate constituted the excystment medium. The presence of these bacterial masses in no wise impaired the excystment-inducing properties of the medium; they tended to conceal the cysts and to render observation difficult.

By means of a blunt needle the cysts were detached from the depression slide, to which they were usually cemented rather firmly by the ectocyst. They were washed in excystment medium and transferred for final excystment to convenient amounts of the medium, usually in groups of twenty. These excystment cultures were then removed to a moist chamber which was kept in an oven at 28°C. Under these conditions approximately 90 per cent. of the specimens emerged within 3-4 hours. The remaining 10 per cent. failed to excyst, even after two days in the medium, though these cysts appeared to be quite normal structurally. At 22°C. 4-6 hours were required for excystment, and the number of specimens that emerged amounted to only 65 per cent. of the total. These percentages are based on experiments with nearly 1500 cysts at 28°C. and 600 cysts at 22°C.

Goodey found that 30°C. is approximately the optimum temperature for the excystment of *Colpoda*; at this temperature many were active at the end of an hour. Haagen-Smit and Thimann, in their analysis of the excystment-inducing substances in hay infusion, allowed 4-4½ hours for the excystment of *Colpoda* at 24°C. Johnson and Evans (1940), using alfalfa infusion, obtained approximately 100 per cent. excystment in *Woodruffia* in 6.5 hours at 30°C. Beers (1945) obtained 100 per cent. excystment in *Tillina* within three hours at 20°C., though Weyer reported that 11½ hours are required for excystment in *Gastrostyla*. This length of time seems excessive, but much depends on the effectiveness of the medium and the physiological state of the cysts. In general, the comparative results indicate that an excystment rate of 90 per cent. within 3-4 hours constitutes a thoroughly satisfactory response on the part of the cysts.

The volume of the excystment medium seems to have little effect on the excystment process in *Didinium*, within reasonable limits. The results are the same, whether 20 cysts are removed to 0.2 cc. in a depression slide or to 2 cc. in a deep watch-glass (Boveri dish). In making the accompanying photographs and drawings 20 cysts were usually mounted in excystment medium on a slide under a cover slip which was supported on half-millimeter cubes of paraffin, and the cover slip was sealed in place with melted vaseline. Under these conditions the percentage of excystment at 28°C.—about 80 per cent.—was usually slightly lower than in open dishes.

It was conclusively demonstrated that the mechanical agitation of detachment and washing in no wise affects excystment. If cysts are detached and tumbled about in the encystment fluid as in actual washing, and then exposed (still in encystment fluid) to a temperature of 28°C., excystment never occurs. Furthermore, detached and washed cysts show a distinctly higher excystment rate than attached cysts from which the old encystment fluid has been removed and merely replaced with excystment medium. The process of detaching and washing brings the excystment medium into direct contact with the ectocyst over its entire surface. Actually, the cyst of *Didinium* is not loosely attached to the substratum at a single point. An abundance of ectocyst material is secreted to fill completely the space between the cyst and its substratum, so that the mature cyst often resembles a piece of tissue over which thick celloidin has been poured to fasten it to a fiber block.

If the *Didinium* cysts were transferred to sterile 0.5 per cent. peptone, 9-15 hours were required for excystment at 28°C. and 18-24 hours at 22°C. Excystment never occurred until the medium was visibly clouded with bacteria! (In spite of repeated washings in sterile peptone and attempts to exclude bacteria from these excystment cultures, bacteria always remained adherent to the sticky ectocyst and thus arrived in the peptone. An effort is being made to devise a method of obtaining bacteria-free *Didinium* cysts.) Similar results were obtained with hay infusion, lettuce infusion and yeast extract. The evidence indicates that excystment in *Didinium* is induced by substances which are produced in the medium through the agency of bacterial metabolism. These findings are in general agreement with those of Weyer, who excysted *Gastrostyla* regularly with a bacterized gelatin medium, though they are quite at variance with the results of Beers (1945) on *Tillina* and of Haagen-Smit and Thimann on *Colpoda*. Further studies are in progress on the rôle of bacteria in the excystment of *Didinium*.

#### THE RESTING CYST

The cyst of *Didinium* is usually a sphere which varies in diameter from 60 to 80 $\mu$  (Plates 9 and 11; Figs. 1 and 13). Its cytoplasm is laden with coarse granules and encloses a horseshoe-shaped macronucleus and several micronuclei. It lacks a vacuole. Like the cyst of *Woodruffia* and *Tillina*, it has three membranes: an outer ectocyst, somewhat glutinous, rather transparent, and of varying thickness; then, a mesocyst which is fairly rigid, tough, dense and laminated, and which undoubtedly constitutes the chief protective component of the cyst; finally, a thin and seemingly tough endocyst which is indistinguishable in the living cyst, but very conspicuous in the later stages of excystment. For the corresponding three membranes of hypotrich cysts Ilowaisky used the terms ectocyst, endocyst and intimocyst, and Beers (1935) followed his procedure. In reality, such terminology is confusing, in that the reader naturally expects the endocyst to be the innermost membrane. It seems advisable, therefore, to designate the innermost, thin membrane of the infusorian cyst as the endocyst, following the practice of Goodey, and to designate the middle membrane (if there is one) as the mesocyst, following Johnson and Evans (1940).

#### THE EXCYSTMENT PROCESS

Selected stages in the process are shown in Plates 9-11, to which all following figure references apply. Plates 9 and 10 are reproductions of photomicrographs of successive stages in the process. They show faithfully the dense granulation of the cytoplasm and the relative size and relations of the component parts of the cyst. Owing to mechanical and other difficulties, they do not show successive stages in the excystment of a single individual. Plate 11, on the other hand, presents a series of camera lucida drawings of one and the same cyst. This plate, therefore, depicts accurately the changes in volume undergone by the cyst throughout the process.

The first observable manifestations of excystment are the initiation of cyclosis,

feeble at first, in the deeper part of the cytoplasm, and the appearance of a small, transparent vacuole quite near the surface. This vacuole, which proves to be a highly important excystment organelle, establishes the polarity of the didinium. It appears at the extreme posterior end of the organism and it remains, fundamentally at least, in this site, unaffected by cyclosis, throughout the excystment process.

Cyclosis becomes gradually more pronounced, and the vacuole increases in size (Figs. 2 and 14). The predominant direction of the protoplasmic streaming is from the equatorial region of the cyst toward the anterior pole (Fig. 14), thence backward along the antero-posterior axis, though there are frequent local disturbances and irregularities in the direction of flow. The vacuole in these early stages of excystment is not a sphere. It is rounded posteriorly, and in this region it conforms to the curvature of the membranes, but its anterior surface is flattened against the cytoplasm (Fig. 2). Its shape indicates that the cytoplasm is relatively viscous and unyielding; otherwise the vacuole would assume a spherical contour. Unquestionably the vacuole owes its progressive growth to the entrance of water through the cyst membranes. To judge by its position, most of the water enters over its posterior surface; otherwise the water must traverse the greater anterior thickness of the cytoplasm, and this seems unlikely. The vacuole as it grows exerts pressure on the cytoplasm anteriorly, and this directed pressure is in turn transmitted anteriorly to the cyst membranes. The cyst itself does not increase appreciably in diameter in spite of the growth of the vacuole. Evidently the membranes are relatively inelastic.

At this stage (Figs. 2 and 14) a slight elevation, which is delimited from the remainder of the protoplasm by a shallow, circular groove, appears at the anterior pole. The granules in it are in constant movement, and the elevation itself is slightly ameboid. This elevation marks the site of the future oral cone and seizing organ, and without doubt it may be regarded at this stage as the primordium of these.

When the vacuole comes to occupy about one-half the volume of the cytoplasm, a bulge appears on the surface of the cyst at or near the anterior pole (Figs. 3 and 15). The membranes become measurably thinner over the bulge as if stretched by pressure from within. A break now occurs in the distended ectocyst and mesocyst, and the protoplasm with its enveloping endocyst, which now becomes clearly visible for the first time, protrudes through the break like a single lobose pseudopodium (Figs. 4 and 16). Sometimes the outer membranes are seen to rupture suddenly, with limited jarring of the cyst as a whole; again, the process of distension and rending of the membranes is so gradual that the act of rupture cannot be perceived as a separate event. The break that develops always takes the form of a fissure (Fig. 20), through which the protoplasm must push its way to the exterior. Only when the cyst wall ruptures does the vacuole assume the shape of a sphere (Figs. 4 and 16).

Thus the relatively inelastic ectocyst and mesocyst are ruptured at the anterior pole by the directed pressure from within. The chief source of this pressure appears to be the ever-growing vacuole itself. There is no evidence that the cyst

membranes themselves possess any kind of inherent polarity, i.e., a weaker, preformed area in which a break would first occur. The presence of an enzyme which attacks the membranes in the region of the cytoplasmic elevation also seems unlikely, in view of the slit-like shape of the break. Furthermore, such an enzyme would have to pass through the endocyst, leaving it unaffected, in order to act on the remaining membranes. Such enzymatic behavior seems improbable, since all the membranes are believed to consist of similar materials (Goodey, 1913). Indeed, the postulation of an enzyme is not needed to explain the facts as observed.

The vacuole continues to grow in size, becoming elongated in the meantime and pushing more and more of the protoplasm through the opening in the cyst wall (Figs. 5, 6 and 17, 18). The torn edges of the cyst wall are forced outward like flaps, which are seen as transverse blurs across the vacuole of Figures 6 and 7. The opening itself is stretched into the shape of an ellipse or even a circle. The cilia appear soon after the rupture of the membranes (Fig. 16 *et seq.*). The endocyst is not a delicate membrane, but a tough, somewhat firm and elastic, protective investment for the animal as it presses through the jagged edges of the opening (Fig. 6, upper right, endocyst in focus and raised above surface of cytoplasm; Figs. 17, 18). The surface of the cytoplasm displays undulatory movements, and the interior is in a state of constant and seemingly disorganized streaming.

Next the posterior end of the organism and the surrounding endocyst begin to slip out of the outer membranes (Figs. 7 and 19), and at this stage the vacuole attains its maximum size. Two simple physical factors seem to be operative in the final extrusion of the organism: first, a tendency on the part of the cytoplasm and the turgid, elastic endocyst to assume the shape of a sphere and secondly, a tendency on the part of the mesocyst to recover its original spherical shape. Finally, the mesocyst and surrounding ectocyst snap away from the endocyst with considerable recoil. At the same moment the endocyst and the cytoplasm become spherical (Figs. 8, 9 and 20A). The torn edges of the mesocyst become approximated, leaving only an angular fissure which sometimes closes completely.

Figure 8, in which the animal is viewed from the side, is of particular interest. The endocyst shows clearly, especially over the anterior half of the protoplasmic sphere. Posteriorly the vacuole appears to be in contact with the plasma membrane. Anteriorly the granular cytoplasm is forced to take the form of a cap, which in optical section appears as an opaque crescent, upon the anterior half of the vacuole. In Figure 9 the didinium is viewed almost from the posterior pole, so that the granular cytoplasm appears as a dark circle around the vacuole. With the low power of the microscope the macronucleus can always be seen in this view as a conspicuous, transparent, curved or looped rod in the cytoplasmic cap, though the objective used in making Figure 9 lacked sufficient depth of focus to show clearly both the vacuole and the macronucleus.

Thus it is seen that through the imbibition of water into the single relatively enormous vacuole the protoplast has by now more than doubled its volume!

To illustrate this point, the protoplast of the resting cyst shown in Fig. 13 had a diameter of  $62\mu$  and hence a volume of nearly 115,000 cubic microns; the protoplast of this same cyst upon emergence from the outer membranes (Fig. 20A) had a diameter of  $82\mu$  and hence a volume of nearly 290,000 cubic microns.

Soon the entire fluid content of the vacuole is discharged abruptly at the posterior end into the space between the cytoplasm and the endocyst. The sudden discharge of the vacuole leaves the entire posterior half of the animal in a much collapsed, furrowed and quite misshapen condition (Figs. 10 and 21). The didinium now begins to rotate slowly within the endocyst as a result of ciliary action, and in scarcely a minute it assumes the smooth, ovoid contour of the adult, free-swimming animal. Up to this point there has been no rotation of the animal as a whole within its cyst membranes. Evidently much of the fluid that is discharged from the vacuole escapes readily to the exterior through the elastic endocyst, which now is smaller in diameter than formerly (cf. Figs. 9 and 10, which are of the same cyst; also 20A and 21). With its oral end directed downward against the endocyst the didinium rotates slowly on its longitudinal axis for some minutes, at times clockwise, again counterclockwise (Figs. 11 and 22). The contractile vacuole appears in its typical position at the posterior end of the animal and begins its rhythmic pulsations. Whether the large excystment vacuole is identical with the contractile vacuole is not entirely clear, though the possibility seems likely. At least the site of origin of the excystment vacuole and its point of final discharge correspond, respectively, to the position of the definitive contractile vacuole and to the location of its point of discharge.

Following the period of continuous rotation within the endocyst, the didinium becomes extremely active. With its oral end, which is still somewhat flattened and not fully developed, it probes, pushes and jabs the entire inner surface of the endocyst unremittingly, displaying incredible persistence and energy (Fig. 23). Its activity, which continues for many minutes up to the moment of its final liberation, is so intense that it cannot be photographed satisfactorily at this stage by ordinary methods (Fig. 12). Soon the endocyst begins to increase rapidly in diameter (Figs. 12 and 24). Next, it becomes thin and slightly wrinkled (Fig. 25); finally, it may be seen to dissolve in the medium, thus liberating the didinium. As Goodey pointed out, the final rapid dissolution of the endocyst indicates that it is digested by an enzyme which is produced by the ciliate well toward the end of the excystment process.

Figure 12 needs a special word of explanation. Here the endocyst appears as a very heavy black line, as if its wall were somewhat thick. In reality, this apparent thickness of the wall merely represents the distance (times 580) through which the endocyst moved peripherally during an exposure of one second. Actually, the wall at this stage is thin and delicate. In Figure 12, to illustrate this point, the endocyst is not spherical, because its thin wall, upon increasing in diameter, has encountered the old outer membranes (at lower left and out of the field) and has been flattened slightly by contact with them.

A didinium upon emergence from its endocyst (Fig. 25) is structurally immature in that its seizing organ is undeveloped and its ciliation is unlike that of

the adult. The anterior girdle of cilia (length of each about  $8\mu$ ) is present as in the adult. Posterior to this girdle there are many rows of shorter cilia (length about  $4\mu$ ) which are meridionally disposed, but the rows end midway between the equator and the posterior pole. During the first half-hour after emergence, the posterior cilia of each row elongate and thus constitute the posterior girdle of the adult, whereas the intervening cilia become shorter and disappear, apparently by resorption. Klein (1930), upon applying his silver method to the adult *Didinium*, observed longitudinal rows of granules ("Relationskörner") over the entire surface of the ciliate. He interpreted the granules of the oral cone as rudimentary basal granules, and the remaining granules (aside from those of the ciliary girdles) as "Protrichocystenkörner." In all probability some of these so-called protrichocyst granules are derived from the basal granules of the short cilia of the immature *Didinium*. A further study of the ciliate before emergence from the endocyst might reveal ciliary antecedents for the granules of the oral cone and posterior segment of the body.

Upon emergence the didinium is content to rest upon the bottom of the dish or to swim sluggishly for an hour or more. Such behavior presents a striking contrast to the intense activity within the endocyst. During this period of reduced activity the oral cone elongates, and the trichites of the seizing organ develop. Only then does the didinium begin its energetic search for food.

#### SUMMARY

Resting cysts of *Didinium nasutum* were induced to excyst by immersion in 0.5 per cent. aqueous peptone solution which had been inoculated 18 hours earlier with wild bacteria from encystment cultures. The evidence indicates that excystment under these conditions was induced by substances which resulted from the metabolic activities of the bacteria.

Excystment is initiated by the appearance of a vacuole in the cytoplasm at the posterior end of the organism. The vacuole increases in volume through the imbibition of water. Thus pressure is exerted from within against the ectocyst and mesocyst, which are forcibly ruptured. With the continued growth of the vacuole, which ultimately reaches such an enormous size that the volume of the organism is more than doubled, much of the protoplasm is gradually extruded from the outer two membranes. The remainder finally slips out, impelled in part by the elastic recoil of the outer membranes. Thus the didinium, now a distended sphere and enclosed in a thin, tough, elastic endocyst, arrives in the external world. Soon the vacuole discharges its contents, and the ciliate for the first time assumes the form of the free-swimming didinium. Finally the endocyst increases in diameter and dissolves in the medium, as if digested rapidly by a powerful enzyme, thus liberating the didinium.

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#### EXPLANATION OF PLATES

##### PLATES 9 AND 10

Photomicrographs of successive stages in the excystment of *Didinium nasutum*.  $\times 580$ . This series of twelve figures is based on five different cysts. Figs. 1-3 show three different cysts; Figs. 4, 5, 7 and 8 are based on a fourth cyst; Figs. 6 and 9-12, on a fifth cyst.

FIG. 1. Resting cyst. Shows ectocyst, mesocyst, and space between mesocyst and cytoplasm; also coarse, dense granulation of cytoplasm.

FIG. 2. Early stage of excystment. Polarity established by conspicuous vacuole at posterior end of cyst.

FIG. 3. Cyst membranes stretched in region of anterior pole by cytoplasmic pressure from within.

FIG. 4. Ectocyst and mesocyst ruptured; cytoplasm protruding; vacuole now a sphere.

FIG. 5. Growth and extension of vacuole anteriorly, with protrusion of more of the protoplasm.

FIG. 6. Continuation of foregoing processes, endocyst shows clearly over anterior surface.

FIG. 7. Posterior end of organism (with enveloping endocyst) slipping out of outer membranes.

FIG. 8. Organism now a sphere, freed of its outer membranes, but still in endocyst.

FIG. 9. Same stage as foregoing, but polar view of vacuole.

FIG. 10. Side view following discharge of vacuole; ciliate in endocyst.

FIG. 11. Rotation of ciliate on its longitudinal axis.

FIG. 12. Rapid increase in diameter of endocyst, just before its dissolution.

##### PLATE 11

Camera lucida drawings of successive stages in the excystment of a single individual of *Didinium nasutum*. This series of figures affords a means of comparing the changes in size undergone by the ciliate at different stages in the process. The time that has elapsed since the cyst was immersed in excystment medium is given for each figure. Temperature,  $28^{\circ}\text{C}$ .  $\times 375$ .

FIG. 13. The resting cyst upon removal to excystment medium.

FIG. 14. Stage of flattened excystment vacuole. Arrows indicate direction of protoplasmic streaming. 2 hrs., 40 mins.

FIG. 15. Rupture of the membranes. 2 hrs., 50 mins.

FIG. 16. Protrusion of cyst contents. 2 hrs., 53 mins.

FIG. 17. Growth of vacuole in diameter and further protrusion of protoplasm. 2 hrs., 55 mins.

PLATE 9

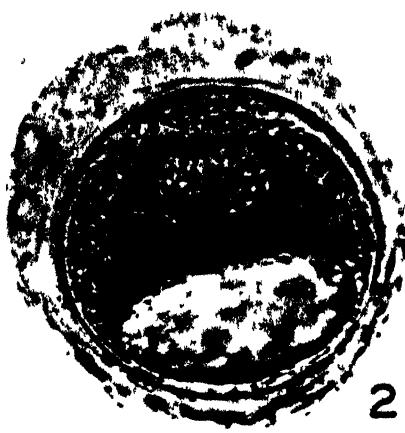




PLATE 10

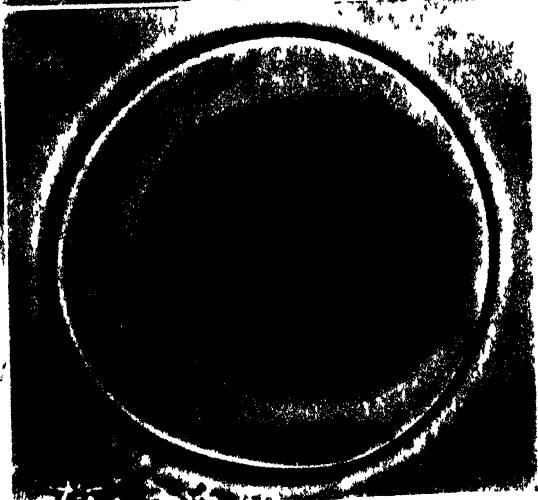
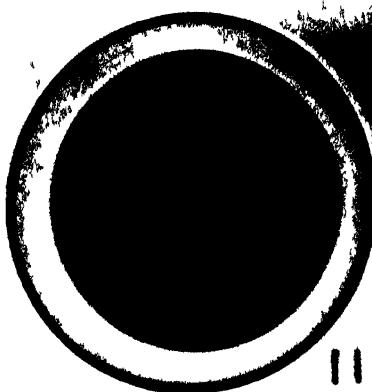
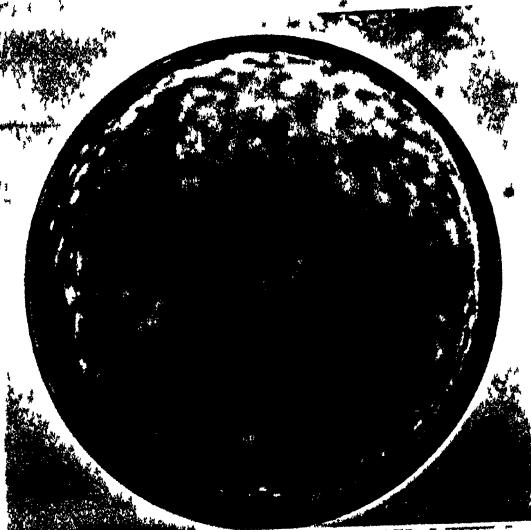




PLATE 11

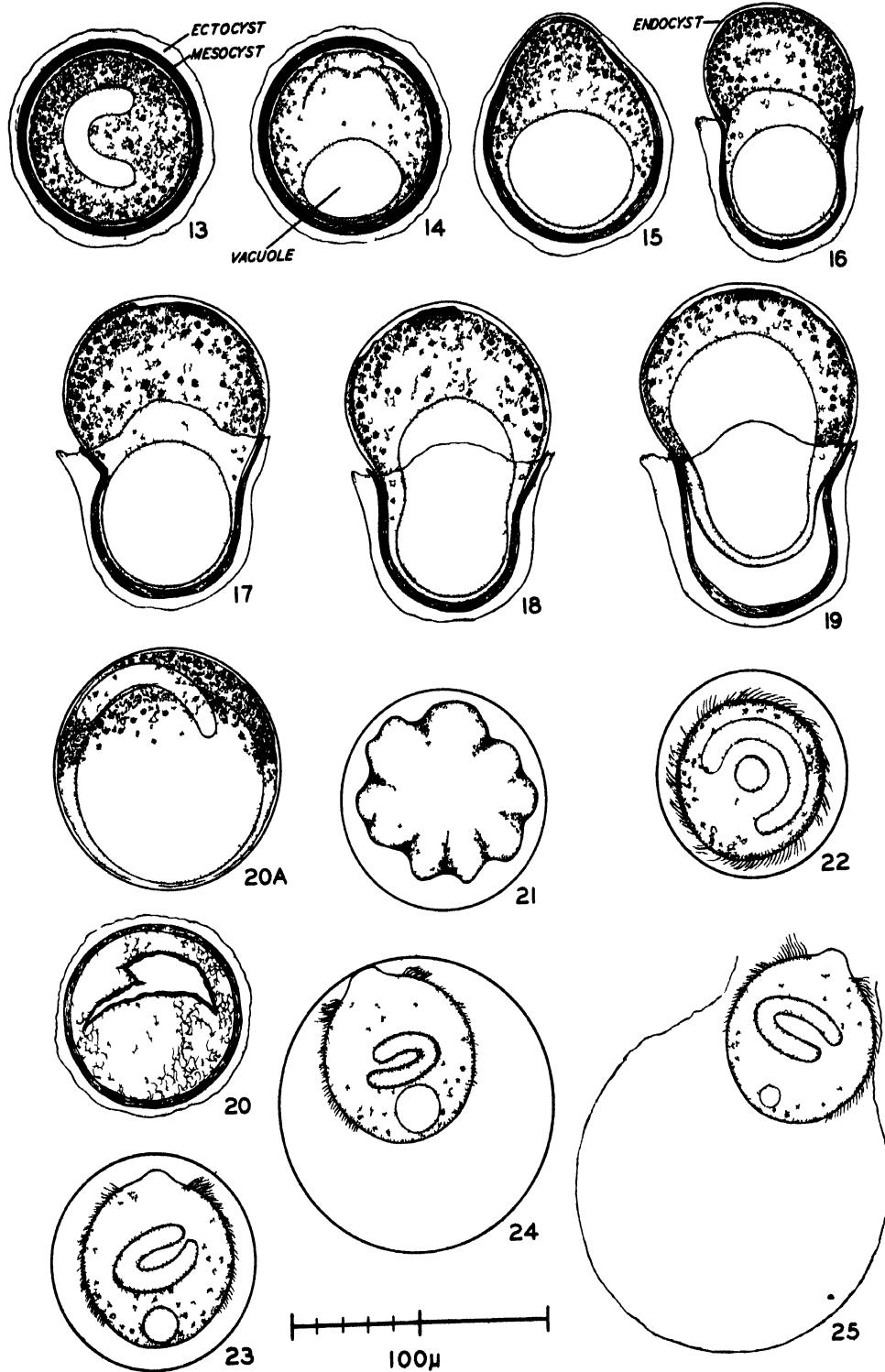




FIG 18 Elongation of vacuole anteriorly 2 hrs, 58 mins

FIG 19 Final escape from outermost membranes 3 hrs

FIG 20 Empty outer membranes, showing slit through which organism emerged

FIG 20A Organism in endocyst, but now freed of outer membranes, vacuole a sphere 3 hrs, 1 min

FIG 21 Organism viewed from posterior pole just after discharge of vacuole 3 hrs, 2 mins

FIG 22 Characteristic shape of adult ciliate now assumed, rotation of organism in endocyst, contractile vacuole present 3 hrs, 3 mins

FIG 23 Didinium still in endocyst, but now attempting to escape, ciliation unlike that of adult 3 hrs, 19 mins

FIG 24 Endocyst increasing in diameter 3 hrs, 20 mins

FIG 25 Dissolution of endocyst in medium and liberation of organism 3 hrs, 21 mins



# SEGREGATION<sup>1</sup> OF OÖPLASMIC CONSTITUENTS

BY DONALD P. COSTELLO

*Department of Zoology, University of North Carolina, and The Marine Biological Laboratory, Woods Hole, Mass.*

WITH PLATE 12 AND ONE TEXT FIGURE

## INTRODUCTION

The segregation of the formed cytoplasmic components in the eggs of certain marine invertebrates, early described by Wilson (1892), Conklin (1905), Lillie (1906) and others, and more recently called "bipolar differentiation" by Spek (1930), is one of the most astonishing and least understood phenomena of development. It is the purpose of the present paper to present a possible explanation of oöplasmic segregation.

This phenomenon is most striking in eggs showing the so-called "determinate" type of cleavage, and may be initiated, in different forms, at the time of, or prior to, germinal vesicle breakdown, during polar body formation, or at fertilization or artificial parthenogenetic activation. Spek (1934b) induced a marked "bipolar differentiation" in the egg of *Asterias*, at a stage when there is normally only a differentiation of cortical from interior protoplasm, by means of hypertonic KCl and hypertonic NaCl.

A classical example of segregation of oöplasmic constituents is found in the egg of the annelid *Nereis limbata*. Before fertilization the egg is flattened somewhat in the polar axis and has a central germinal vesicle around which is arranged a double row of large oil droplets (fig. 1). The other cytoplasmic components (yolk spheres, mitochondria, neutral-red-staining granules, other microsomes, and the hyaline protoplasm) are approximately uniformly distributed, except in the cortical layer, where tiny granules of a jelly-precursor are so arranged as to simulate alveoli and their contents. At fertilization or activation the jelly-precursor is released through the vitelline membrane, and swells to form the thick enveloping layer of jelly characteristic of the fertilized egg. A short time later the germinal vesicle breaks down (fig. 2) and there begins a movement of the cytoplasmic components, together with some flowing of the hyaline protoplasm, so that the oil droplets and most of the yolk spheres come to lie in the vegetal hemisphere, and most of the clear protoplasm and smaller cytoplasmic granules are found in the animal hemisphere. The small, spherical mitochondria (which can be demonstrated in the living condition, by Janus green, only after the egg has been divested of its membrane) form a ring below the animal pole.

<sup>1</sup> I see no objection to speaking of this phenomenon as oöplasmic or cytoplasmic segregation (cf. Needham, 1942), since the name differentiates it from embryonic segregation as defined by Lillie (1927). Lillie (1906) refers to the process as polarization, but recognizes that it is essentially a re-polarization of the egg constituents. Wilson (1925) calls it pre-localization and in one instance, at least, segregation. The term "bipolar differentiation" is not a satisfactory description of all types of oöplasmic segregation.

The neutral-red-staining granules form, just above the equator, a layer which spreads out as a very thin cortical zone toward the mitochondrial ring. The oil droplets move nearer to the vegetal pole than the yolk spheres, and slowly fuse with each other until, in later development, only four remain, one in each of the large endodermal cells. Obviously there is some hyaline protoplasm in the interstices between the granules in all regions.

Segregation of the cytoplasmic constituents along the polar axis is apparent in each blastomere of the 4-cell stage in *Nereis*. At the third cleavage, the micromeres, separated at the animal hemisphere, are oil-free and practically yolk-free. At the 16-cell stage, the oöplasmic substances contained in the four tiers of blastomeres are quite different. In the egg of *Chaetopterus* (Lillie, 1906) the essential segregation of the oöplasmic components has been accomplished by the time of the metaphase of the first maturation division. In *Nereis limbata* cytoplasmic segregation proceeds at a relatively slow rate, somewhat more than two hours elapsing between germinal vesicle breakdown and the third cleavage (at 20°C.). It is much more rapid in the egg of the ascidian *Styela partita*.

Cytoplasmic segregation can be readily observed in the egg of *Nereis limbata* although this egg does not possess the natural pigment described by Spek (1930, 1933) for the egg of *Nereis dumerilii*. A variety of vital dyes, including those used by Spek (1934a) render the cytoplasmic segregation more apparent. Spek believes that, after "bipolar differentiation" has taken place, the animal hemisphere of the egg (at the four-cell stage, for example) contains granules which are alkaline in reaction (as determined by vital staining with indicator dyes) in an alkaline animal plasm. The vegetal hemisphere consists of an acid yolk plasm, of which the yolk spheres are the chief constituent. In the equatorial region are some acid yolk spheres in a medium of alkaline protoplasm. The so-called alkaline region gives rise to the ectodermal structures of the larva, the acid "yolk-plasm" region produces the endoderm, and part of the border region containing yolk spheres in the alkaline plasm produces the prototroch (an ectodermal structure). Raven (1938) agrees with Spek's description of the staining reactions of the egg of *Nereis dumerilii* and gives additional data on the staining properties of the strata of the centrifuged egg.

That the staining substances which participate in oöplasmic segregation are identical with the different egg substances that separate from each other under the influence of centrifugal force was demonstrated by Raven (1938) for the egg of *Nereis dumerilii*, and by Ries (1939) for the egg of *Aplysia*. Unfortunately both authors failed to state the centrifugal force employed, giving only the speed of the centrifuge and the duration of treatment, and omitting the effective radius. From the speeds indicated, we may be certain that the forces employed were small. Costello (1936) performed a series of experiments on the eggs of *Nereis limbata* with strong centrifugal forces (up to 270,000 times gravity) and concluded that with the vital dyes employed (neutral red and nile blue sulphate), at truly vital concentrations, the hyaline protoplasm of the *Nereis* egg does not stain. Only by using these dyes at concentrations high enough to be injurious was staining of the hyaline protoplasm accomplished. The strong centrifugal

forces sedimented granules that were normally present in the "hyaline zones" of the less strongly centrifuged eggs. All coloration of the egg by the dyes was associated with discrete granules or vacuoles, many of which approach the limits of microscopical visibility, but all movable by centrifugal force if the eggs remain in the living condition and are centrifuged for a sufficient period with an appropriate force. That there may be two different kinds of hyaline protoplasm of different specific gravity in a given egg is a possible interpretation of the two hyaline zones described for the centrifuged *Nereis* egg by Costello (1938) and for the centrifuged egg of *Ilyanassa* by Clement (1935). But that these types of hyaloplasm are associated with bipolar differentiation or stain differently in the living condition has not been demonstrated.

Needham (1942) has summarized briefly the data relating to staining differences of segregated cytoplasmic substances in marine eggs. To this summary should be appended a statement that these staining differences are of no significance for morphogenesis if, as is generally accepted, the visible cytoplasmic inclusions, displaceable with centrifugal force, may be shifted to any position with respect to the egg axis without altering normal development. Only differences of staining capacity within the hyaline protoplasm would be of significance for morphogenesis.

It is obvious from the observations of Raven (1938) on the egg of *Nereis dumerilii*, Ries and Gersch (1936) and of Ries (1937, 1939), on the egg of *Aplysia*, and from my own observations (1938, 1940) on centrifuged eggs of *Nereis limbata*, that the stratification of the cytoplasm produced by centrifuging is quite different from that brought about by the normal segregation of oöplasmic substances. The most striking difference is that in the centrifuged *Nereis* egg, oil and yolk are driven to opposite ends of the axis of stratification, whereas they are closely associated in the vegetal hemisphere of the non-centrifuged egg after the onset of cytoplasmic segregation. Therefore, as was early recognized by Conklin for the egg of *Styela*, the phenomenon of oöplasmic segregation cannot be accounted for on the basis of an action of gravity sedimenting egg inclusions of different specific gravity.

Spek (1930, 1934a, 1938) speaks of oöplasmic segregation as a "self-cataphoresis," but there is no evidence for flow of electric current through the cell and the process cannot be considered true electrophoresis. He recognizes the importance of external ions. Harrison (1940) makes a significant suggestion when he says, "The sorting out process requires energy and, according to some, even intelligence. It may be related in some way to the bioelectric potentials of both the ovum itself and the surrounding maternal tissue." But he has obviously not arrived at a conclusion as to the mechanism. Earlier in the same paper he remarks that substances, either combined with proteins or separate, would tend to concentrate near one or the other pole of the egg according to their electric charge. Again no mechanism is mentioned, and Harrison is presumably thinking in terms of Spek's hypothesis of "self-cataphoresis." No satisfactory physico-chemical explanation of these localization movements has as yet been presented in the literature.

## THE THEORY OF "DIFFUSION EFFECT"

The present theory was suggested by Schrader's (1944) discussion of the possible rôle of diffusion potentials in the mechanism of mitosis. It is based upon Teorell's (1935, a, b, c, d, 1937a, b) studies of diffusion potentials. The formulation of Teorell was apparently arrived at independently by Meyer and Sievers (1936) and is a generalized formulation of an equilibrium similar to the Gibbs-Donnan equilibrium, the latter, which applies where indiffusible ions are present (Teorell, 1935b), constituting a special case of the former. This is a type of propulsive force not ordinarily considered in cyto-mechanics. Rashevsky (1940) has suggested in numerous papers the importance of diffusion forces in cell division, etc., but has not visualized these forces in terms of the Teorell diffusion potential. He has not discussed oöplasmic segregation.

The following discussion is an attempt to apply the Teorell diffusion effect to an explanation of a simple type of oöplasmic segregation—that of segregation of materials along the polar axis. In the form presented, the theory could not, without modification, account for a more complex type of oöplasmic segregation, such as that found in the egg of *Styela*, where the primary polar segregation is followed by the formation of the posterior yellow crescent, etc.

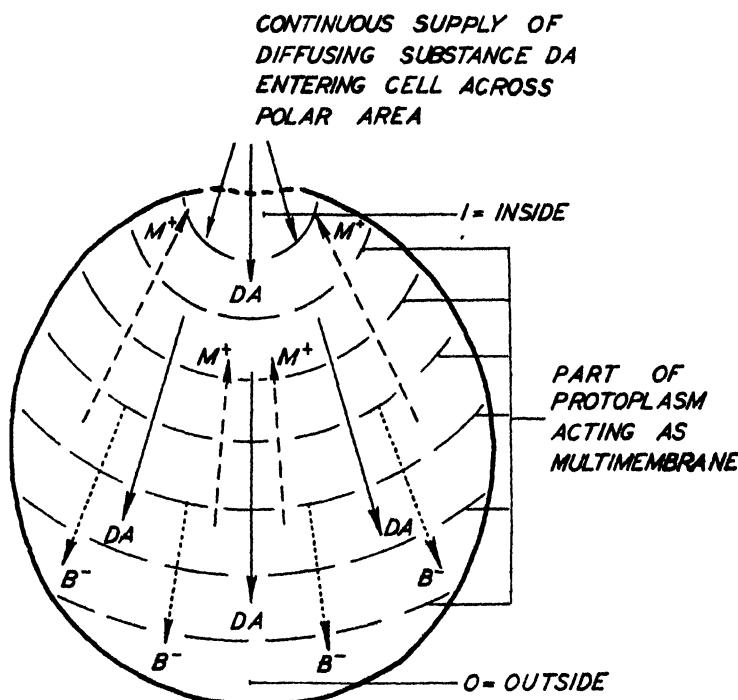
The system set up by Teorell (1935b, 1937b) is as follows:

Across a permeable boundary (membrane)  $m$  there is assumed to be present a constant difference in concentration of either the cation  $D^+$  or the anion  $A^-$ . The maintenance of this condition, accomplished, for example, by a continuous addition of the substance DA to the rather small volume  $i$  (= inside), causes DA to act as a "diffusion agent" which steadily diffuses across  $m$  into the part  $o$  (= outside). In  $o$  a fixed, constant composition is maintained by keeping the volume large.

It was predicted that the continuous steady diffusion of DA was bound to influence the distribution of other electrolytes present, denoted by  $M'B'$ ;  $M''B''$ , etc., which were not participating in any active diffusion as was DA. These ions were called "passive ions." The membrane is permeable to these passive ions. It was assumed that the  $D^+$  ions had a higher mobility within the boundary than the  $A^-$  ions, producing an electrical potential across the boundary  $m$ . Starting with the initial state of equal concentrations of  $M^+$  and  $B^-$  on the two sides of  $m$ , the electrical potential causes an inward migration of  $M^+$  and an outward migration of  $B^-$ . Finally the concentration gradients become sufficiently large to balance the electrical gradient and the system approaches a steady state. Thus the  $M^+$  ions become accumulated and the  $B^-$  ions diminish in amount inside. See text fig. 1. Accordingly a diffusion of one electrolyte may produce an accumulation or impoverishment inside of other cations or anions, depending upon the mobilities of the ions of the diffusing agent. This effect upon ionic distribution is called the diffusion effect.

Teorell (1937b) has given the equation for the distribution of the passive ions in terms of the final diffusion potential. For the theoretical considerations of the present paper no calculations will be presented.

Originally (Teorell, 1935b) the theory dealt with the steady state conditions of ionic distribution in aqueous systems where one electrolyte was diffusing, creating a diffusion potential which brought about a redistribution of other ions. The concepts were later extended (1935c) to indicate that any electrically charged particle, regardless of its size, is subjected to the influence of diffusion potentials present in the system, positively charged particles being moved toward the negative part of the diffusion potential field and negative particles in the opposite direction. It was emphasized that the "diffusion effect" upon ionic, colloidal, etc., distribution is not an electrolysis or cataphoresis effect, because no external



TEXT FIGURE 1. Proposed biological analogue of Teorell scheme, employing "multimembrane" or thick diffusion layer to bring about segregation of colloidal particles  $M^+$ ,  $B^-$  within cell.

E. M. F. is applied and no current is flowing. The diffusion effect is due to exchange of charged particles arising from differences in mobility of the ions of the diffusing substance. Cases of accumulation and impoverishment were experimentally demonstrated for proteins and colloidal suspensions as well as for inorganic ions.

In applying the Teorell diffusion effect as an explanation of cytoplasmic segregation, a number of questions arise. These are (1) the necessity for, and the nature of, the membrane; (2) the origin of the directed diffusion gradient within the cell; (3) the effect of a small volume in  $o$  (outside) and  $i$  (inside) rather than a large volume; (4) the magnitude of the diffusion effect in relation to the known

rate of segregation of the oöplasmic constituents and in relation to the magnitude of the charge on such colloidal particles as are represented by the oöplasmic components; (5) the possible nature of the diffusing substance (DA). These will be discussed in order.

(1) In regard to the necessity for a membrane, Teorell (1935a) studied ionic concentration distribution within a diffusion layer itself, recognizing that a membrane as such is not requisite. Utilizing an agar plug in a glass tube did not, however, prove to be convenient, so he adopted a multimembrane arrangement consisting of a series of cellophane or collodion sheets clamped between suitable washers. While this was not equivalent to a homogeneous diffusion layer from the kinetic point of view, it proved to be so when the time factor disappeared, i.e., in a stationary state. Dixit (1938) states that the presence of a membrane is not necessary for the existence of a Gibbs-Donnan equilibrium.

It seems apparent that, in the Teorell system, anything can function as a membrane that differentially retards one ion of the diffusing substance as compared with the other, to set up the diffusion potential.<sup>2</sup> A diffusion potential would be produced if there were a steady diffusion (concentration gradient) from one part of a cell to another, if the intervening medium acted as a membrane in retarding one or the other members of the ion pair. Any other electrically charged particle would be moved in such a system, positively charged particles being moved to the negative part of the diffusion potential field and negative particles in the opposite direction.

It will be assumed that in order to get from the initial condition of concentric or slightly polarized localization of cytoplasmic constituents to the final condition of bipolarly segregated constituents by means of the diffusion effect something extending throughout most of the egg must be acting as a membrane. This may be part of the hyaline protoplasm itself. The "membrane" can be most easily visualized as a framework through which the cytoplasmic granules are free to move. That such a framework actually exists, in some eggs at least, seems to be demonstrated, despite the fact that in recent years there has been a tendency to consider protoplasm as a fluid system. In the egg of *Chaetopterus*, Lillie (1906) demonstrated a "spongy area" and certain other areas by means of low centrifugal force. That these areas disappeared under higher centrifugal forces is perhaps evidence that the framework is neither extensive nor rigid, but probably of low viscosity, though not as low as the viscosity of the medium which is free to pass through it. In the fertilized egg of *Arbacia* there is a cortical gelated region in which the pigment granules are largely accumulated (Wilson, 1926). Sol-gel changes within cells are well known. Conklin (1916, 1917), largely on the basis of the formation of giant polar bodies on eggs of *Crepidula* undergoing maturation divisions in the centrifuge, held that there is a framework of resistant protoplasmic strands extending throughout the cell. Even if this tenuous framework does not serve as the "membrane," this function being performed by some other part of the protoplasm, it is important to stress the

<sup>2</sup> The normal difference in mobility of the ions of a substance is sufficient, but this mobility difference may be accentuated by certain types of membranes (Teorell, 1937b)

fact that the existence of such a framework within the cell would not necessarily hinder the movement of the other formed cell components with which the theory deals.

The assumptions then are that  $i$  is the region of the cell near the animal pole,  $o$ , the region of the cell near the vegetal pole, and  $m$ , a multimembrane or thick diffusion layer extending throughout most of the cell, through which the oöplasmic substances are free to pass under the influence of the diffusion potential. The diffusing substance (DA) must then continuously enter the  $i$ - $m$ - $o$  system from the external medium or originate near one of the poles. The movement of the charged particles takes place essentially within the diffusion layer. The volumes of  $i$  and  $o$  are negligible by comparison with that of  $m$ .

(2) There are several mechanisms by which a directed (or polarized) concentration gradient could arise in a cell. This is essentially the same problem as the origin of gradients in the axial gradient theory of Child. We are here concerned with a diffusion (concentration) gradient of an ionizable substance (DA) that can give rise to a diffusion potential. Whether any one of the mechanisms listed below actually obtains in living cells is a question that cannot be answered at present. The possible mechanisms for the origin of the diffusion gradient are:

- a). Directed diffusion from outside the cell due to the presence of a local area of the cell membrane with a special permeability.
- b). Growth additions in the ovarian egg, materials being laid down around a polar axis, with marked gradients along this axis, by which a visible polarity is established in the egg (Harrison, 1940). Presumably the diffusing substance in this concentration gradient becomes bound to a protein framework at a very early (pre-segregation) stage, perhaps even before the formation of the oöplasmic inclusions. A special stimulus would later be required to free the bound substance from the protein to set up the diffusion potential. The original entry of the substance which becomes bound might be by mechanism (a) described above.
- c). A liquid crystal structure in the oöplasm involving long polarized units. Diffusion between these units might proceed at a greater rate in one axis than in others. Unidirectional diffusion could be obtained in such a system only if some mechanism similar to (a) above were also operating, or if the egg always oriented with one pole in contact with the substratum, the other pole free in the external medium from which DA enters.

- d). A special diffusion center, very excentrically placed in the egg cell, acting as a source of DA. The poles of the spindle (centrosomes) are assumed to be diffusion centers in Rashevsky's (1938) theory of mitosis. Consideration of the centrosome as the diffusion center initiating oöplasmic segregation leads us into all the complexities of Boveri's centrosome theory of fertilization. I am inclined to discard this possibility on the grounds (1) that the egg centrosome present during the early history of the egg is not sufficiently excentrically located; (2) that the sperm-center does not cause a redistribution of oöplasmic substances in all forms. It may be recalled, however, that in the egg of *Styela* the sperm does initiate oöplasmic movements. In other cases secondary factors, such as high

cortical viscosity, may prevent such an effect. This is a possibility to be reserved for future consideration.

Since mechanism (a) may be of significance in three of the four possible mechanisms described above, I shall discuss this in more detail:

The special permeability across a localized area of the cell membrane would be assumed to be due to a special morphology of this area. In this region an increase in permeability would be assumed to occur at some definite time in relation to the segregation movements of the oöplasm. This might be either in the ovary, when the egg is removed from the body of the female to sea water, at the time of germinal vesicle breakdown, fertilization or activation, depending upon the egg species. The localized area across which the increase in permeability would take place could be, theoretically, at either the animal or vegetal polar region. Actually, there are several reasons for suggesting that the animal pole region is the more likely of the two. This is usually the free end of the attached ovarian egg, but the primary reason is that in this region of the cell, in eggs of many forms, there is visible a distinct polar defect. This has been described by Lillie (1906) for the egg of *Chaetopterus* as a defect in the ectoplasm where the endoplasm comes to the surface under the external delicate membrane. Let us examine the history of the polar defect in the egg of *Chaetopterus* to see whether its behavior is in accord with that required by the theory.

In the ovarian egg of *Chaetopterus* the endoplasm comes to the surface at two places, (1) a small area in the center of the animal pole, where the polar bodies later form (the polar, or ectoplasmic defect); (2) over a large portion of the vegetal hemisphere. The latter area lacks the thin vitelline membrane. The rupture of the germinal vesicle, brought about by the introduction of the egg into sea water, initiates a series of movements of the egg substances by means of which they attain their definitive positions during the prophases of the first maturation division. This series of movements which takes place with reference to the polar axis of the egg produces a topographical arrangement corresponding in many essential respects to the future embryonic areas. Before the rupture of the germinal vesicle the ectoplasm covers the upper two-thirds of the egg; as soon as the germinal vesicle breaks down, it flows toward the vegetal pole, and, even before the first maturation spindle has become fixed at the pre-delineated animal pole, it has completely overflowed it, thus covering up the exposed endoplasm. The membrane accompanies the ectoplasm and so covers the entire egg. At the end of the polarization period the ectoplasm around the center of the vegetal hemisphere is as thick as elsewhere. The original opening in the ectoplasm at the animal pole meanwhile has become enlarged and the outer end of the maturation spindle is fixed there. At the first cleavage, the polar bodies and ectoplasmic defect are transmitted entirely to the CD blastomere; at the second cleavage to the D blastomere; and at the third cleavage the polar defect is found on the 1d blastomere. In later development this ectoplasmic defect is the place of formation of the apical organ with its flagella. In eggs differentiating without cleavage, which lack the apical flagella, there is a corresponding lack of a polar defect, this spot having been covered by overflow of the ectoplasm.

Lillie considers the ectoplasmic defect of the egg of *Chaetopterus* to correspond to the animal polar area of the egg of *Dentalium* (Wilson, 1904) and to the animal pole-plasm of the egg of *Rhynchelmis* (Vejdovsky, 1888-92). I am inclined to view the ectoplasmic defect as consisting not only of the endoplasm which is at this point in contact with the vitelline membrane, but also of the pre-delineated portion of the membrane or cortex which determines the position of the maturation spindle, and likewise determines the direction of flow of the endoplasm as the area enlarges. In the egg of *Cumingia* (fig. 4) there is a visible wrinkling of the membrane in this region shown in a section of a fixed egg. It may be pointed out that in certain eggs (*Patella, Nereis*) the apical flagella are not limited to D-quadrant micromere derivatives. The correlation between this and the distribution of the polar defect during cleavage has not been made.

From this morphological description of the *Chaetopterus* egg, it appears that the animal polar defect, being smaller, could give rise to a better-directed diffusion gradient than the larger vegetal hemisphere defect which disappears during the process of cytoplasmic segregation. However, the two defects at opposite poles may tend to maintain diffusion gradients balanced against each other until some physiological condition is altered. However, absence of the vitelline membrane on the vegetal hemisphere is not characteristic of marine eggs.

The rôle of the polar defect in oöplasmic segregation could be tested by studies of egg fragments. If the polar defect is of significance, only polar fragments of eggs cut before the onset of oöplasmic segregation would carry on the process. Spek (1934a) considers a local permeability change in the region of the polar bodies of significance for the "bioelectric potential" causing "cataphoresis" of the oöplasmic substances.

I discarded the possibility that bipolar movement of substances in the egg might be obtained if the polar defect region of the cell membrane were the diffusion potential membrane  $m$ , because, first, the cell membrane does not satisfy the Teorell assumptions in being permeable to the cell inclusions, and second, polar but not bipolar segregation would, at most, result.

(3) The assumption that  $i$  and  $o$  are small and that  $m$ , the diffusion layer, extends throughout most of the cell leads us away from the original assumptions on which Teorell based his theory. However, since this seems to be essentially similar to the multimembrane arrangement used by Teorell (1935a), it must be assumed that the basic principles are still applicable.

(4) The magnitude of the diffusion effect in relation to the known rate of segregation of the oöplasmic constituents is unknown. Teorell (1937a) obtained a rapid "chemotaxis" of proteins and colloidal particles in the presence of a diffusion potential of only some millivolts. He attributes this marked effect to the high charge carried by such particles. The rate of cytoplasmic segregation in various marine eggs is as follows: *Chaetopterus*, 15 to 30 minutes, depending upon the temperature and some unknown factors (Lillie, 1906); *Nereis limbata*, about 90 minutes (it is difficult to state when the process ceases in *Nereis*); *Styela*, about 45 minutes. In the last-named form, the initial downflow of yellow and clear protoplasm to the vegetal hemisphere takes place so rapidly that it can be seen in the living egg. The movement begins with sperm penetration, and

the illustrations of Conklin (1905) show a considerable movement within the first five minutes following that event. Later movements to form the typical crescents are considerably slower.

It is a question whether the oöplasmic substances would be moved by the diffusion potential at a rate comparable to that actually observed. We cannot at present test the theory by direct application of values for concentrations, diffusion potential, or electrokinetic charges of the particles, since these are unknown. The rapidity of the process in certain forms appears to favor a chemical change rather than a diffusion process. Therefore mechanism (b) which involves the sudden release (presumably by chemical change) of a bound electrolyte along a steep concentration gradient seems worthy of consideration.

(5) The nature of the diffusing substance (DA) is a matter about which very little can be stated. There is a possibility that the substance is potassium chloride. With KCl, Spek induced "bipolar differentiation" in the egg of *Asterias*. Potassium chloride also induces *Chaetopterus* eggs to complete their maturation (Mead, 1898) and to undergo differentiation without cleavage (Lillie, 1902, 1906). However, it is important that the diffusing substance should show a considerable difference in mobility of its ions in the "membrane," in order to set up a diffusion potential of any great magnitude.

#### SIGNIFICANCE OF OÖPLASMIC SEGREGATION

Wilson (1925) reviews the processes of oöplasmic segregation (as seen in living, unstained material) for the eggs of *Paracentrotus* (*Strongylocentrotus*), *Rhynchelmis*, *Clepsine*, *Styela*, *Myzostoma*, *Physa*, *Lymnaea*, *Planorbis*, *Dentalium* and *Sternaspis* and concludes that they show a common general type of prelocalization that is attained at different periods during precleavage. The process occurs relatively late in the sea urchin (after completion of the maturation divisions) or ascidians (during extrusion of the polocytes following fertilization), at an intermediate period in *Myzostoma* (beginning before maturation, but not completed until afterward) and very early in *Dentalium* (long before maturation, before the egg has completed its full growth in the ovary). The chief differences between the so-called "mosaic" eggs and the "regulative" eggs are merely (1) a different morphological relation of the cleavage pattern to certain localized oöplasmic materials, (2) the time relationship between determination and such developmental events as fertilization, cleavage, and gastrulation, and (3) the regulative capacity (cf. Gersch and Ries, 1937; Gersch, 1937). It is thus possible to arrange the various egg species in a nearly continuous series between widely separated extremes, all conforming to the mosaic principle in greater or less degree and yet all showing some regulative properties.

The visible movement of suspended oöplasmic granules of various kinds are viewed by Wilson (1925) as only the external signs of underlying processes in the apparently structureless fundamental ground-substance or hyaloplasm that for the most part escape direct observation. This means that the morphogenetic substances are not the visible cytoplasmic components, but perhaps localized physico-chemical modifications of this hyaloplasm, or hormones, enzymes, or the like, localized within it.

The theory proposed to explain oöplasmic segregation would account for the segregation of *invisible* charged substances, such as hormones and enzymes, as well as for the segregation of the larger visible formed cytoplasmic inclusions. It therefore provides a basis by which developmental potencies can be normally associated with visible cytoplasmic areas (as demonstrated in the egg-fragment studies on *Dentalium* by Wilson, 1904) and yet not associated with visible cytoplasmic inclusions (as demonstrated by centrifuging experiments). If this theory is valid, we could thus have, as the result of the movement of charged particles in a diffusion potential field, both the visible stratification of the suspended oöplasmic substances and the parallel but invisible polarization of the "formative stuffs."

#### SUMMARY

A theory is suggested to account for the segregation of oöplasmic constituents in terms of the Teorell "diffusion effect."

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## PLATE 12

## EXPLANATION OF FIGURES

All figures except figure 4 are photomicrographs of living *Nereis* eggs. Magnification, figs. 1-3,  $\times 270$ ; 4,  $\times 500$ ; 5-6,  $\times 465$ .

FIG. 1 *Nereis* egg before insemination, polar view. Note central germinal vesicle, large oil droplets, cortex of jelly-precursor.

FIG. 2. *Nereis* egg 28 minutes after insemination, polar view. Germinal vesicle has broken down. Cortex has disappeared, leaving perivitelline space. Invisible jelly is external to cell membrane.

PLATE 12

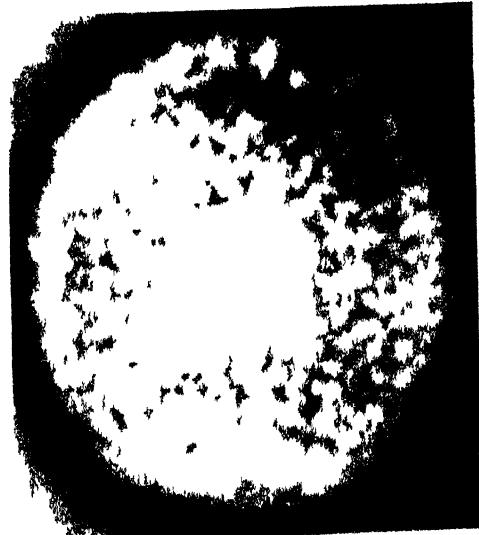
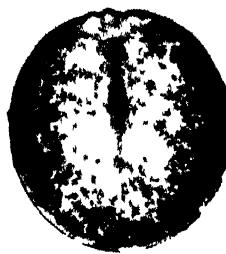
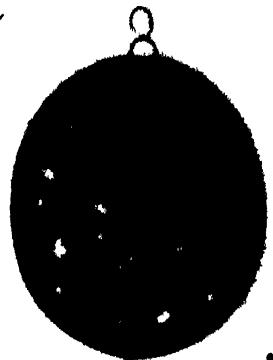
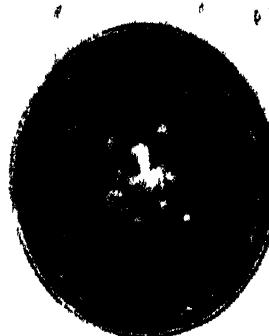
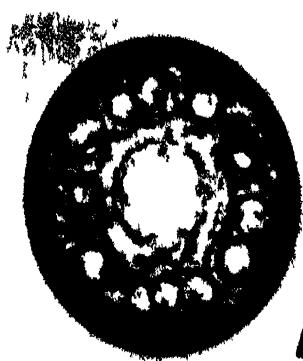




FIG. 3. *Nereis* egg 59 minutes after insemination, and 54 minutes after beginning of treatment with alkaline NaCl to remove membrane. Side view, with polar bodies toward top of page. Oöplasmic segregation, as reflected by the position of the oil droplets, has begun.

FIG. 4. Section (in axis of spindle) of fixed and stained egg of *Cumingia tellinoides*, unfertilized, at resting stage of first maturation metaphase. Note wrinkling of vitelline membrane at animal pole.

FIG. 5. Surface view of animal pole region of *Nereis* egg 45 minutes after insemination, showing polar area free of yolk spheres.

FIG. 6. Same, 58 minutes after insemination, showing two polar bodies in polar area.



## ON HOMOEOSIS IN DROSOPHILA

BY CLAUDE A. VILLEE

*Department of Zoology, University of North Carolina*

WITH ONE TEXT FIGURE

This paper presents first a discussion of the relation between homoeosis and homology and a reply to some remarks made by Ferris (1943) and Boyden (1943) on a previous paper (Villee, 1942b). The second part of the paper presents some new data on the quantitative nature of the difference between the aristata-pedia alleles of *D. melanogaster* derived from a study of the effect of temperature treatments on the compound  $ss^a/ss^{aB}$ .

The term "homoeosis" was introduced by Bateson (1894) for those variations which consist of the assumption by one member of a meristic series of the form or characters proper to other members of the series. Bateson cited many cases of homoeotic variations in the vertebrae and ribs of vertebrates—in man, in gorillas, orang-utans, chimpanzees, sloths, dogs, cats, snakes and frogs. The phenomenon is also found widely in the Arthropods—as cases of abnormal development or abnormal regeneration occurring in nature. The presence of a tarsus in place of an antenna has been found in a saw-fly, *Cimber axillaris* (Kraatz, 1876) and in a bee, *Bombyx variabilis* (Kriechbaumer, 1889). The development of a chela in place of a maxilliped was reported by Bateson (1890) in the crab, *Cancer pagurus*. Wheeler (1896) found an antenna-like structure growing out of the coxa of the right prothoracic leg of the midge *Dilophus tibialis*, and Bethe (1896) described a crab, *Carcinus maenus* in which one of the abdominal swimmerets was replaced by a large thoracic walking leg. The development of an antenna in place of an eye was found in *Palinurus*, the spiny lobster, by Milne Edwards (1864) and in a stomatopod, *Squilla pallida*, by Giesbrecht (1910). Several instances of the occurrence of additional oviducal openings in the crayfish *Astacus* were summarized by Bateson (1894). Korschelt in his monograph on regeneration (1927) cites several reports of various crustacea in which the mandible was replaced by a claw-tipped thoracic leg or by a swimmeret, or in which the thoracic legs were replaced by swimmerets.

The first example of experimentally produced homoeotic regeneration was described by Herbst (1896) in the decapod *Palaeomon*, in which an antenna-like structure developed in place of an extirpated eye-stalk. Krizinecky (1914) obtained the same regeneration of an antenna in place of a destroyed optic ganglion in the larva of the mealworm, *Tenebrio*. The regeneration of tarsus-like structures in the place of extirpated or destroyed antennae was produced by Przibram in the praying mantis (1909), in certain beetles (1919a), and in saw-flies (1919b) and by Cuenot (1921), Brecher (1924) and Borchardt (1927) in the walking stick, *Dixippus*.

A number of groups of arthropods show hereditary homoeosis in nature. The Lepidopteran family Zygaenidae in which the metathoracic wings are completely

similar to the mesothoracic wings was described by Lebedinsky (1925); and the aberrant Dipteran, *Termitoxenia*, which lives commensally in the burrows of termites, and whose wings are much reduced and intermediate between wings and halteres (balancers), was described by Wasmann (1900). Bezzi's monograph (1916) describes several Hymenoptera, Neuroptera, Strepsiptera and Coccids in which the metathoracic wings are reduced to structures resembling the halteres of Diptera, and other parasitic Hymenoptera, Orthoptera and Homoptera in which the mesothoracic wings are haltere-like.

Several homoeotic mutations have been found in *Drosophila*. The earliest ones were found in *D. melanogaster* but as new species are investigated intensively, homoeotics similar to the ones in *melanogaster* are discovered. Bithorax is known in *melanogaster*, *pseudoobscura*, *affinis*, and *athabasca*; aristapedia in *melanogaster*, *simulans*, *pseudoobscura* and *willistoni*; tetraptera, tetraptera, proboscipedia and ophthalmomopedia have been found so far only in *melanogaster*.

The first homoeotics described were bithorax (bx, 3—58.8\*) and bithoraxoid (bx<sup>d</sup>, 3—58.8+) (Bridges and Morgan, 1923). In bithorax there is a more or less complete change of the metathorax into a structure resembling a mesothorax. As in most homoeotics, the extent of the change is variable, often unequal on the two sides, and sometimes overlaps the wild type. The sides and dorsal surface of the metathorax are covered by a bristle-bearing structure which resembles the mesonotum and scutellum. The halteres are changed in various ways: some are swollen and hairy, others are inflated and sack-like, resembling the wings of the mutant "inflated," and others are enlarged, flattened and wing-like, bearing veins. Bithoraxoid, which is not an allele of bithorax, resembles it, having inflated halteres but a less well-defined hairy structure on the dorsal surface of the metathorax. Astauroff (1927, 1929) described a third mutant, tetraptera (ttr., 3—51.3), not an allele of either bithorax or bithoraxoid, in which a similar change occurs. The phenotypes of tetraptera included a complete series from a normal haltere to a completely developed metathoracic wing.

In aristapedia (ss<sup>a</sup>, 3—58.5) (Balkashina, 1929) the arista, the plumose tip of the antenna, is replaced by a tarsus-like organ consisting of up to five segments. The various phenotypes form a series from a reduced antenna of only two segments to a structure of seven or eight segments which consists of the three normal antennal segments plus a four- or five-segmented tarsus complete with claws, pulvilli and a saw-toothed bristle.

Bridges and Dobzhansky (1933) described proboscipedia (pb, 3—47.7) in which the oral lobes, which normally form the proboscis, are replaced by a pair of tapering, jointed appendages. These extend laterally and are tipped with strong, claw-like bristles or with a heavy branched bristle resembling an arista. The appendages vary considerably in length, size, distinctness of segmentation and in the terminal bristles. The rest of the mouthparts are also modified: the pseudotracheae disappear completely, the labrum and maxilla change shape, and the maxillary palpus fuses with the maxilla, so that the mouth-

\* 3—58.8 means that the bithorax gene is located at locus 58.8 in chromosome 3.

parts of a proboscipedia fly resemble somewhat the biting mouthparts of lower insects.

The mutant *tetraltera* (tet, 3—48.5) (Goldschmidt, 1940; Villee, 1942a) changes the normal mesothoracic wing through a large intermediate series into a small haltere-like appendage. In addition, the mesonotum and scutellum may be reduced or missing, changing the appearance of the dorsal mesothorax to that of a metathorax.

Another hereditary condition in *Drosophila*, which may or may not be truly homoeotic, is the development of an antenna-like structure instead of an eye or in combination with a rudimentary eye. All intermediates between a small, hairy knob and a segmented, palpus-like structure of three or four segments may be found. Instead of a true homoeotic change of an eye anlage into an antenna, this may be the result of a splitting early in development of the antennal disc, one part of which forms the antenna in its usual place and the other part of which encroaches on the eye region and suppresses partially or completely the development of the eye. Waddington and Pilkington (1943), investigating the development of ophthalmopedia, found that the palp developed from an abnormal fold in the optic disc in the late larva. A detailed study of the development of other mutants of this type is needed. This condition has been reported as a high allele of *kidney* (Goldschmidt, 1929, 1940; Gottschewski and Ma, 1937; Valadares, 1938), as an allele of *Lobe* (Goldschmidt, 1940) as ophthalmopedia (Gordon, 1936) and as the mutant *erupt* (Glass, 1944).

These mutants have several features in common distinguishing them more or less from other mutants:

1. The phenotypes are very variable, forming a series from a normal, unchanged organ to a complete, homoeotic replacement of it by a homologous structure.
2. There are large variations in penetrance, i.e., in the percentage of flies homozygous for the mutant which show the character phenotypically.
3. The degree of symmetry in the expression of the character varies greatly; animals with complete symmetry are rare.
4. There is a low degree of right-left correlation in the expression of the character (Astauoff, 1930).

The investigators who have worked with these homoeotic mutants have all remarked upon the evolutionary importance of these single mutations which produce a change of major taxonomic importance. Bridges and Dobzhansky (1933) said: "In no representative of the order Diptera is there found a type with the structure of the mouthparts even remotely similar to that present of proboscipedia. Only outside the order Diptera, among insects possessing mouthparts of the biting type, structures of the labium comparable to that in proboscipedia are common." The presence of one instead of two pairs of wings is one of the distinguishing characteristics of the order Diptera, yet bithorax, bithoraxoid and tetraptera produce flies with two pairs of wings. Goldschmidt (1940) pointed out the taxonomic parallel to the *tetraltera* mutant shown by the aberrant Dipteran *Termitoxenia*. In comparing the various phenotypes produced by

the tetraptera mutant with the *Termitoxenia* types given in Kenner's (1940) review of the group, it was found (Villee, 1942a) that one of the phenotypes intermediate between a wing and a haltere, which resembled neither very clearly, was almost identical with the *Termitoxenia* wing. The mutant tetraptera thus produces a fly whose wings resemble those of a member of a completely different family of Diptera.

Goldschmidt (1940) emphasized the importance of the homoeotic mutants as models of macroevolutionary changes. To produce the oral lobe type of mouthparts from the biting mouthparts of lower insects, it is not necessary to postulate the gradual accumulation of many micromutations for the oral lobe type; instead a single mutation affecting early embryological processes may produce the new type in a single step. Animals with the intermediate types of mouthparts necessary on the Neo-Darwinian hypothesis could not have fed themselves sufficiently to have survived. Mutations controlling the speed of differentiation, the gradients of segmentation, the nature of the evocator or the time of induction of the different discs, could produce at one step changes in the phenotype of the segmental organ of macroevolutionary importance.

The mutants bithorax, tetraptera and tetraptera demonstrate the homology of wing and haltere, a homology which had been proven previously on morphological grounds. The aristapedia phenotype indicates the homology of antenna and tarsus, supporting the classical idea that the insect antenna is a homologue of the mouthpart-leg series of segmental appendages, and contradicting the theory that the insect antenna is a separate pre-oral structure not homologous to the mouth part-leg series as suggested by some comparative anatomists. The phenotypes of proboscipedia indicate the homology of the oral lobes with the labium of other insects.

Ferris (1943) has criticized these views of Dobzhansky and Goldschmidt, which were reviewed by the author previously (1942b), on two points: 1. The insect morphologists have settled the homology of the oral lobes and contributions from genetics are unnecessary. 2. There are hundreds of flies with intermediate types of mouthparts (intermediate between biting and sucking) with which they survive nicely. In a counter critique Goldschmidt (in press) answered Ferris and showed: 1. that morphologists are not agreed on the homology of the oral lobes-- a number of competent entomologists (Peterson, Newport and Gershfeld) consider the paraglossae to be the homologue of the oral lobes and Snodgrass in his text leans to this view; and that the experimental transformation of one organ into another is the supreme criterion of homology; and 2. that while flies do show many variations in the details of their mouthparts, the principle of a dipteran labium is present from the beginning and the change of the primitive biting labium into the dipteran sucking labium was probably accomplished in a single step. In a recent morphological study of the mouthparts of Mecoptera and Diptera, Imms (1944) concluded that the labella (oral lobes) are formed of the two-segmented labial palpi, both paraglossae and glossae being absent. That Imms should have felt it necessary to make this study would indicate that he did not agree with Ferris that the homologization had been proven "beyond a

reasonable doubt" by previous morphological work. The entomologist Kemner (1940) in his review of the *Termitoxenia* group believes that the haltere-like wings of this group were produced in a single step also.

In a recent review of homology and analogy Boyden (1943) made a sharp distinction between special homology, "an essential similarity in the structure and development and in the relative position and connections of the corresponding parts of the bodies of different organisms," and serial homology, "structural similarity in a series of appendages along the chief axis of the body of one individual." The distinction is valid and should be kept in mind in discussions of homology. However, Boyden dismisses serial homologies as being mere intra-individual structural expressions and of no use as evidence in tracing relationships. Hubbs (1944) rejected this viewpoint and presented evidence of the usefulness of serial homologies in phylogenetic research. The present author regards special and serial homologies as two facets of the same underlying genetic relationship. In arthropods (and vertebrates) the existence of serially homologous sets of appendages and their alterations in various animals is important in tracing phylogenies. The ancestral arthropod is believed to have had a many-segmented body, with each segment bearing a pair of appendages, probably biramous, all serially homologous and each specially homologous with the appendage of the similar segment in other forms. When a mutation occurs affecting the development of one of those pairs of appendages so that it develops (perhaps according to the mechanism suggested by Goldschmidt, 1940) as a new phenotype—a tarsus, antenna, cheliped, swimmeret, or what-not—that new appendage is still serially homologous with the other appendages of that animal and specially homologous to the appendage of that same segment in other animals, even though it may look quite different and have a different function. Homologous organs retain certain potentialities, or "competences" in Waddington's terminology, to develop in several different ways which they inherit from their ancestors. This is demonstrated clearly by the homoeotic mutants. Only organs with these competences can so develop. The fact that the developmental reaction systems involved in the development of antenna and tarsus are similar enough so that a single gene difference (e.g., *aristapedia*), perhaps by altering the timing of the determination of the anlage or by altering the chemistry of the evocator, can cause an antennal primordium to develop as a tarsus is a strong indication of their close homology and common descent. Perhaps in time it may be possible to define homologies accurately in terms of the inherited competences of various anlagen.

None of the investigators of homoeotic mutations have supposed that they involve changes in the relative positions of organs, e.g., the transposition of tarsi to the mouth region or the antenna (cf. Boyden, loc. cit., p. 235-236). The homoeotic mutants affect the development of the primordium of some member of a segmental series so that it assumes the characteristics of some other member of the segmental series. The fact that in *aristapedia* the tarsi of the legs are sometimes defective does not mean that they have been transposed to the antennae, but only that the *aristapedia* gene affects their development as well as

that of the antennae. It would theoretically be possible (although genetically very difficult, since the genes are located in the same chromosome only a few units apart) to set up a *Drosophila* stock combining bithorax and tetraptera and have a fly with haltere-like mesothoracic appendages and wing-like metathoracic appendages! But this would not, of course, mean that a reciprocal transposition had occurred, rather that under the action of the two homoeotic genes, a wing primordium developed into a haltere and a haltere primordium developed into a wing.

Several previous reports (Villee, 1942a, 1943a, b, 1944a, d, 1945a) have discussed some aspects of the phenogenetics of homoeosis in *Drosophila*. The expression of either of the aristapedia alleles may be increased by exposing developing larvae to cold (14.4°C.), or decreased by exposing developing larvae to heat (29° or 35°C.). The most effective time to begin either heat or cold treatments is after 4 days of development at room temperature (25°C.), although treatments begun anywhere from 1 to 5 days after development begins have some effect. The expression of bithorax, however, is increased by heat treatments and decreased by cold treatments given to developing larvae. Exposing proboscipedia larvae to 15°C. alters development so that only arista-like appendages form in the place of oral lobes, and exposing them to 29°C. shifts development so that only tarsus-like appendages form in the place of oral lobes. A discussion of these results and their relation to the theory of the action of homoeotic mutants proposed by Goldschmidt (1938, 1940) has been given previously. (See especially 1944a, 1945a.)

The experiments with aristapedia indicate that a quantitative difference exists between the two alleles. The decrease in the velocity of development produced by the cold treatment enables the aristapedia-Bridges gene, working at a lower rate for a longer time, to produce a phenotype similar to that which aristapedia produces at normal temperatures working at a higher rate for a shorter time. The aristapedia alleles may be producing some "tarsus substance" as Waddington (1940) suggested or they may be affecting the time of maturation of the antennal discs as suggested by Goldschmidt (1938). That it is not the cold itself but the increase in the total time of development caused by lower temperatures which is effective is shown by the fact that "partial starvation" of aristapedia-Bridges larvae, using the Beadle (1938) and Khouvine (1938) technique, which increases the total time of development, also increases the expression of aristapedia-Bridges.

The quantitative nature of the difference between the aristapedia alleles is emphasized by experiments with compounds of the two genes. A system of coding had been devised previously (1943a) so that a single number could represent the effect of a given treatment on a whole group of flies. The code number is the sum of the products of the percentage of flies of each type times the type number (1 to 8, cf. loc. cit.). The code number, therefore, lies between 100 (if 100% of the group were type 1) and 800 (if 100% of the group were type 8). Aristapedia-Bridges flies under control conditions have a mean code number of

$163.1 \pm 24.2$  and aristapedia flies, almost all of which are type 8 under control conditions, have a code number of about 795. The compound of aristapedia and aristapedia-Bridges ( $ss^a/ss^{aB}$ ) has a code number of  $364 \pm 16.3$  under control conditions. The phenotypes ranged from type 2 to type 6; most of the flies were type 3 or 4. Larvae of the genotype  $ss^a/ss^{aB}$  responded to heat and cold treatments similarly to homozygous  $ss^a$  and homozygous  $ss^{aB}$ ; heat treatments decreased the expression and cold treatments increased the expression. The most

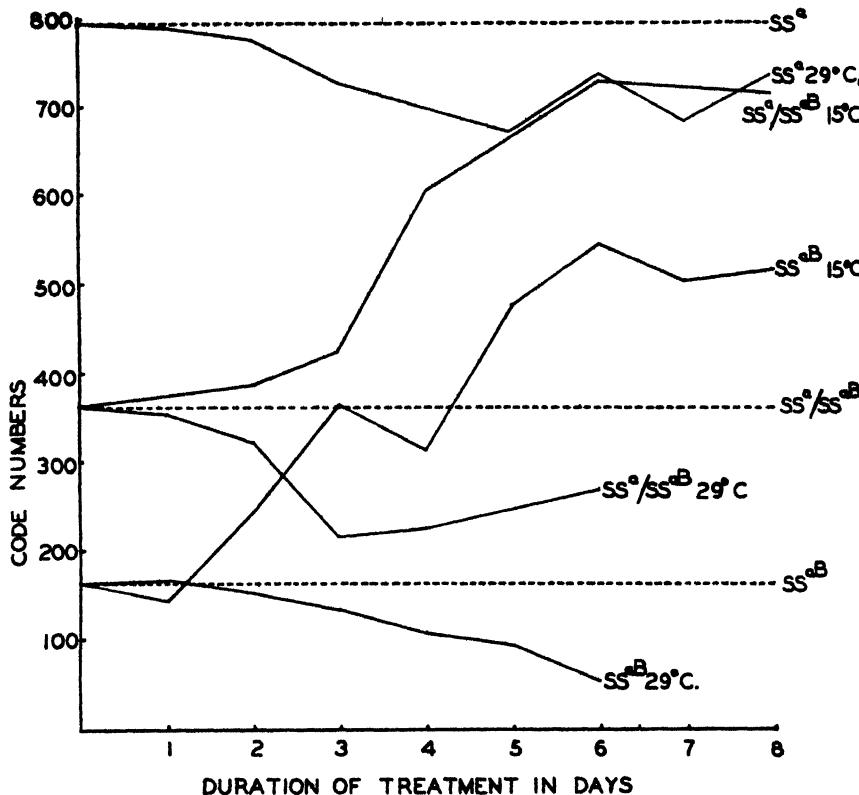


FIG. 1. Comparison of the effects (given in code numbers) of  $15^\circ C.$  and  $29^\circ C.$  treatments begun three days after development begins on the phenotypes of aristapedia ( $ss^a$ ), aristapedia-Bridges ( $ss^{aB}$ ) and the compound of the two ( $ss^a/ss^{aB}$ ) with the duration of the treatment. The dotted lines are the control (untreated) phenotypes.

effective times for beginning the temperature treatments were again 3 or 4 days after development begins. The effect of temperature treatments begun three days after development begins on  $ss^a$ ,  $ss^{aB}$  and  $ss^a/ss^{aB}$  is compared in figure 1. It can be seen that the compound  $ss^a/ss^{aB}$  reacts qualitatively like the homozygous forms; there is simply a quantitative difference between the three stocks.

Heat and cold treatments given to the larvae of aristapedia flies of *D. pseudoobscura* (Villee, 1944d) gave results similar to those in *D. melanogaster*. The experiments indicate that the aristapedia genes in the two species have identical

effects on their respective developmental reaction systems. To express this sort of relationship between genes the term "homophenic" has been proposed (Villee, 1944b). Homophenic genes are ones which affect the same part of the developmental reaction system in the same way at corresponding times in development in two species and thus produce identical phenotypes. The "vermilion" gene of *Drosophila* is probably homophenic with the "aa" gene of the flour moth, *Ephestia*. The "aa" gene of *Ephestia* changes the normal black eye (AA) to red. The vermilion gene of *Drosophila* changes the normal dark red eye to light red. Becker and Plagge (1937) showed that extracts from the dark eyes of AA *Ephestia* when injected into the body cavity of vermilion *Drosophila* would change the eye color to wild type (dark red), just as extracts of wild type *Drosophila* will change vermilion eyes to the darker wild color. The substances and the reaction chains involved seem to be the same in the two species and the vermilion gene and the "aa" gene are probably homophenic.

A phenogenetic study (Waddington, 1943) of genes which affect the legs, dachs, dachsous, four-jointed and combgap, showed that they produce their effects by altering the relative growth rates of the parts. The growth effects of the four genes differ both in the parts of the body which they affect and the kinds of effects produced. All the genes are pleiotropic and affect body and wing size and venation as well as having some effect in shortening or distorting the legs. Since temperature changes were shown to affect homoeotic mutants by affecting the rate of growth, a study was made of the interactions of these genes with some of the homoeotic mutants (Villee, 1944c, 1945b) by making the appropriate crosses. Two types of interactions were found: certain of the homoeotic mutants increase the effect the growth rate genes normally have in shortening the legs, and certain of the growth rate genes increased or decreased the effect the homoeotic genes normally have on their respective structures.

Both aristapedia and aristapedia-Bridges increase the reduction of the tarsus normally produced by dachs and four-jointed so that the tarsi are extremely short, consisting of only one or two segments. A lesser interaction occurs between aristapedia or aristapedia-Bridges and dachsous or combgap: the tarsi of such combinations vary from three to five segments. Proboscipedia does not increase the shortening of the tarsi normally produced by the growth rate genes.

Dachsous combined with aristapedia-Bridges produces a marked increase in the expression of the gene, and thus has an action similar to that of cold treatments. The genes dachs or four-jointed increase slightly the expression of aristapedia-Bridges, but combgap has no effect on the phenotype of this allele. The expression of aristapedia is reduced by the action of four-jointed or combgap to as low as type 4 or 5, and by the action of dachs or dachsous to as low as type 6 or 7. A change in the growth rate induced by these genes thus has the same effect on a homoeotic mutant as one induced by exposing the larvae to changes in temperature.

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WHEELER, W. M. 1896 An antenniform appendage in *Dilophus tibialis*. Arch. Entw.-mech. **3**: 261-268.

PUBLICATIONS IN THE FIELD OF SCIENCE FROM THE  
UNIVERSITY OF NORTH CAROLINA (1934-1944)\*

DEPARTMENT OF BOTANY

JOSEPH EDISON ADAMS

Ph.G., Coll. of Pharm., Columbia Univ., 1925; Ph.Ch., Ibid., 1927; B.S., Univ. of Michigan, 1929; M.A., Columbia Univ., 1932; Ph.D., Univ. of California, 1935.

Instructor in Botany, Univ. of North Carolina, 1935-1936; Assistant Professor, 1936-1941; Associate Professor, 1941-.

1939 Vegetative Regeneration in the West Coast Manzanitas. (Abst.) Journ. E. M. Sci. Soc. **55**: 244.

1940 A Procedure for Staining Filamentous Algae and Fungi on the Slide. Stain Tech. **15**: 15-16.

A Systematic Study of the Genus *Arctostaphylos* Adans. Journ. E. M. Sci. Soc. **56**: 1-62.

RUTH ELLIS ALLEN

B.S., Virginia Polytechnic Inst., 1939; M.A., Univ. of North Carolina, 1944.

Assistant in Botany, Univ. of North Carolina, 1940 (spring)-1944.

1944 Four Ascosporic Species of *Aspergillus* Found on Tea Leaves. Journ. E. M. Sci. Soc. **60**: 171-181, pls. 68-69.

WALTER LANE BARKSDALE

B.A., Univ. of North Carolina, 1937; M.A., Ibid., 1941.

Assistant in Botany, Univ. of North Carolina, 1934-1935, 1936-1938.

1936 Some Notes on Orchids of the Piedmont and Western North Carolina. Pamphlet, 12 pp. Chapel Hill, N. C.

1937 The Occurrence of *Liparis Loeselii* and *Habenaria bracteata* in North Carolina. Journ. E. M. Sci. Soc. **53**: 137-138, pl. 14.

1938 The Pedicellate Species of *Trillium* Found in the Southern Appalachians. Journ. E. M. Sci. Soc. **54**: 271-296, pls. 27-35.

GEORGE ANDREW CHRISTENBERRY

B.S., Furman Univ., 1936; M.A., Univ. of North Carolina, 1938; Ph.D., Ibid., 1940.

Assistant in Botany, Univ. of North Carolina, 1936-1940.

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\* This bibliography is a continuation of the one prepared by Alma Holland for this JOURNAL in 1934. A few titles omitted from the earlier bibliography have been entered here. The present lists have been furnished for the most part by the various schools or departments concerned and have been edited to some extent. Unfortunately, some research which has been done by our faculty cannot be reported here, since it has been done at the request of the government on behalf of the war effort and has not yet been released for publication.—A. H. B.

1938 A Study of the Effect of Light of Various Periods and Wave Lengths on the Growth and Asexual Reproduction of *Choanephora cucurbitarum* (Berk. and Rav.) Thaxter Journ E. M. Sci. Soc. 54: 297-310, pls 36, 37 Abst in U. N. C. Record No. 335 12

1940 A Taxonomic Study of the Mucorales in the Southeastern United States Journ E. M. Sci. Soc. 56: 333-366, pls 13-19 Abst in U. N. C. Record No. 359 13.

WILLIAM CHAMBERS COKER

B. S., South Carolina Coll., 1894, Ph. D., Johns Hopkins Univ., 1901, Student, Univ. of Bonn, Germany, 1901-1902

Associate Professor of Botany, Univ. of North Carolina, 1902-1908\*, Professor of Botany, 1908-1920; Kenan Professor of Botany and Director of the Arboretum, 1920-1944, Head of the Department, 1908-1944, Kenan Research Professor, 1944-

1935 A Remarkable New Rhododendron Journ E. M. Sci. Soc. 51: 189-190, pls 53, 54  
Parasitic Flowering Plants of North Carolina (Abst) Journ E. M. Sci. Soc. 51: 249

1936 Inter-relationships of the Saprolegniales (Abst) Proc. 6th Intern. Bot. Cong. (Amsterdam) 1: 268-270

1937 Professor Duncan Starr Johnson Science 88: 510-512  
Blastocladiales, Monoblepharidales, and (with Velma Matthews) Saprolegniales North Amer. Flora 2, pt 1 1-76 (bibliog., pp. 69-76, with J. H. Barnhart)  
(With H. R. Totten) The Trees of the Southeastern States, 2nd ed. 417 pp. The University of North Carolina Press, Chapel Hill

1938 (With Mary S. Taylor) Filmy Ferns in the Carolinas Science 88: 402  
(With Jane Leitner) New Species of *Achlya* and *A. podachlyoides* Journ. L. M. Sci. Soc. 54: 311-318, pls 38, 39  
A Filmy Fern from North Carolina Journ. E. M. Sci. Soc. 54: 349-350, pl. 40, figs. 5 and 6, and pl. 41

1939 (With Leland Shanor) A Remarkable Saprophytic Fungoid Alga Journ. L. M. Sci. Soc. 55: 152-165, pls 22, 23  
A New *Scleroderma* from Bermuda Mycologia 31: 624-626, one text fig  
New or Noteworthy Basidiomycetes Journ. E. M. Sci. Soc. 55: 373-386, pls 34-44

1941 (Edited) Letters from the Collection of Dr. Charles Wilkins Short Journ. E. M. Sci. Soc. 57: 98-168

1942 Notes on Rare Hydnoms Journ. E. M. Sci. Soc. 58: 94-97, pls 13-16 and 4 text figs

1943 *Magnolia cordata* Michaux Journ. E. M. Sci. Soc. 59: 81-88, pls 17-20 and 6 text figs  
(With Alma Holland Beers) The Boletaceae of North Carolina (96 pp., 66 pls.) The University of North Carolina Press, Chapel Hill

1944 The Woody Smilaxes of the United States Journ. E. M. Sci. Soc. 60: 27-69, pls 9-39

\* In the earlier bibliography this date was erroneously given as 1907, as it has appeared in Who's Who and elsewhere

The Smaller Species of *Pleurotus* in North Carolina. Journ. E. M. Sci. Soc. **60**: 71-95, pls. 40-52.

1945 (With H. R. Totten) The Trees of the Southeastern States, 3rd ed. (419 pp., illus.) The University of North Carolina Press, Chapel Hill.

## JOHN NATHANIEL COUCH

B.A., Univ. North Carolina, 1919; M.A., Ibid., 1922; Ph.D., Ibid., 1924.

Instructor in Botany, Univ. North Carolina, 1922-1925; Assistant Professor, 1925-1928; Associate Professor, 1928-1932; Professor, 1932-; Head of the Department, 1944-.

1935 New or Little Known Chytridiales. *Mycologia* **27**: 160-175, 64 text figs.

*Septobasidium* in the United States. Journ. E. M. Sci. Soc. **51**: 1-77, pls. 1-44.

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A New Saprophytic Species of *Lagenidium*, with Notes on Other Forms. *Mycologia* **27**: 378-387, 40 text figs.

An Incompletely Known Chytrid: *Mitochytridium ramosum*. Journ. E. M. Sci. Soc. **51**: 293-296, pl. 62.

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A New Fungus Intermediate between the Rusts and *Septobasidium*. *Mycologia* **29**: 665-673, 30 text figs.

The Formation and Operation of the Traps in the Nematode-Catching Fungus, *Dactyliella bembicodes* Drechsler. Journ. E. M. Sci. Soc. **53**: 301-309, pl. 27. Abst. in U. N. C. Record No. 323: 9.

1938 A New Chytrid on *Nitella*: *Nephrochrytrium stellatum*. Amer. Journ. Bot. **25**: 507-511, 34 text figs. Abst. in U. N. C. Record No. 335: 10.

The Genus *Septobasidium*. (302 pp., 114 pls., 60 text figs.) The University of North Carolina Press, Chapel Hill.

Observations on cilia of aquatic Phycomycetes. (Abst.) *Science* **88**: 476.

A New Species of *Chytridium* from Mountain Lake, Virginia. Journ. E. M. Sci. Soc. **54**: 256-259, pl. 24.

1939 A New *Conidiobolus* with Sexual Reproduction. Amer. Journ. Bot. **26**: 119-130, 53 text figs.

Technic for Collection, Isolation, and Culture of Chytrids. Journ. E. M. Sci. Soc. **55**: 208-214.

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Further Studies on Infection of Scale Insects by *Septobasidium*. (Abst.) Third Intern. Cong. for Microbiol., Abstracts of Communications, pp. 217-218.

1940 Notes on *Septobasidium* from Mexico. (Abst.) Journ. E. M. Sci. Soc. **55**: 223-224.

1941 (With Vera K. Charles, J. G. Harrar, and J. J. McKelvey, Jr.) A Fungous Parasite of the Mealy Bug. (Abst.) *Phytopathology* **31**: 5.

A New *Uredinella* from Ceylon. *Mycologia* **33**: 405-410, 12 figs.

The Structure and Action of the Cilia in Some Aquatic Phycomycetes. *Amer. Journ. Bot.* **28**: 704-713, 58 figs. Abst. in *Journ. E. M. Sci. Soc.* **57**: 204-205.

1942 (With Alma J. Whiffen) Observations on the Genus *Blastocladiella*. *Amer. Journ. Bot.* **29**: 582-591, 66 figs.

Studies on Lower Fungi with Particular Reference to Sex and Nutrition. (Abst.) *Amer. Phil. Soc. Year Book* 1941: 153-154.

A New Fungus on Crab Eggs. *Journ. E. M. Sci. Soc.* **53**: 158-162, pls. 18, 19.

1943 The Rediscovery of *Nadsonia*, a Yeast with Heterogamic Conjugation. (Abst.) *Journ. E. M. Sci. Soc.* **59**: 118.

1944 The Yeast *Nadsonia* in America. *Journ. E. M. Sci. Soc.* **60**: 11-16, pl. 1.

#### HIDEN TOY COX

B.S., Furman Univ., 1936; B.A., *Ibid.*, 1937; M.A., Univ. of North Carolina, 1939.

Assistant in Botany, Univ. of North Carolina, 1937-1938; Teaching Fellow, *Ibid.*, 1938-1940; Assistant, 1940-1941.

1939 A New Genus of the Rhizidiaceae. *Journ. E. M. Sci. Soc.* **55**: 389-397, pls. 45, 46, and one text fig. Abst. in *U. N. C. Record* No. 347: 14.

#### FRANCES KATHERINE FOUST

B.A., Woman's Coll., U. N. C., 1936; M.A., Univ. of North Carolina, 1938.

Assistant in Botany, Univ. of North Carolina, 1936-1938.

1937 A New Species of *Rozella* Parasitic on *Allomyces*. *Journ. E. M. Sci. Soc.* **53**: 197-204, pls. 22, 23. Abst. in *U. N. C. Record* No. 335: 12-13. 1938.

#### WILLARD CHAPPELL HEWITT

B.A., Univ. of North Carolina, 1934; M.A., *Ibid.*, 1939.

Assistant in Botany, Univ. of North Carolina, 1932-1935; Research Assistant in the Medical School, 1939-1942.

1939 Seed Development of *Lobelia amoena*. *Journ. E. M. Sci. Soc.* **55**: 63-82, pls. 10-13. Abst. in *U. N. C. Record* No. 347: 14-15.

(See also under Dr. R. L. Holman, Department of Pathology, Medical School.)

#### ANDREW GEORGE LANG

B.A., Miami Univ. (Ohio), 1932; M.S., Cornell Univ., 1933; Ph.D., Univ. North Carolina, 1936.

1936 Spermatogenesis in *Marsilea*. *Journ. E. M. Sci. Soc.* **52**: 307-336, pls. 31-35. Abst. in *U. N. C. Record* No. 311: 7-8.

## DENNIS H. LATHAM

Graduate Student, Univ. of North Carolina, 1934-1935.

1935 *Achlya recurva* Cornu from North Carolina. Journ. E. M. Sci. Soc. **51**: 183-187, pl. 52.

## JANE GAGE LEITNER

B.A., Coker College, 1935; M.A., Univ. of North Carolina, 1938.

Assistant in Botany, Univ. of North Carolina, 1935-1938, and summer, 1939.

1938 (With W. C. Coker) New Species of *Achlya* and *Apodachlya*. Journ. E. M. Sci. Soc. **54**: 311-318, pls. 38, 39.

1939 (With J. N. Couch and Alma Whiffen) A New Genus of the Plasmodiophoraceae. Journ. E. M. Sci. Soc. **55**: 399-408, pls. 47, 48.

## ANDREW CLARK MATHEWS

B.A., Univ. of North Carolina, 1928; M.A., Ibid., 1931; Ph.D., Ibid., 1939.

Teaching Fellow in Botany, Univ. of North Carolina, 1929-1932.

1939 The Morphological and Cytological Development of the Sporophylls and Seed of *Juniperus virginiana* L. Journ. E. M. Sci. Soc. **55**: 7-62, pls. 1-9. Abst. in U. N. C. Record No. 347: 12-13.

## VERA MILLSAPS

B.S., North Carolina Coll. for Women, 1915, M.S., Univ. of North Carolina, 1924; Ph.D., Ibid., 1936.

1936 The Structure and Development of the Seed of *Paulownia tomentosa* Steud. Journ. E. M. Sci. Soc. **52**: 56-75, pls. 4-6.

1940 Structure and Development of the Seed of *Cynoglossum amabile* Stapf & Drumm. Journ. E. M. Sci. Soc. **56**: 140-164, pls. 4-6.

## EARL HOLLAND NEWCOMER

B.A., Columbia Univ., 1932; M.A., Univ. of California, 1935; Ph.D., Pennsylvania State Coll., 1938.

Assistant Professor, Univ. of North Carolina, 1941-.

1941 A Colchicine-Induced Tetraploid *Cosmos* and Some Comparisons with its Diploid Progenitor. Journ. Hered. **32**: 160-164, figs. 7, 8.

A Colchicine-Induced Tetraploid Cabbage. Amer. Nat. **75**: 120.

1943 An *F<sub>2</sub>* Colchicine-Induced Tetraploid Cabbage and Some Comparisons with its Diploid Progenitor. Journ. E. M. Sci. Soc. **59**: 69-72, pls. 13-16.

Studies in the Nature of the Clonal Variety. IV. Cytological Studies of Bud Sports of McIntosh, Stark and Baldwin Apples. Mich. St. Coll. Agric. Exp. Station Tech. Bull. No. 187. (23 pp., 117 figs.)

1945 Induced Parthenocarpy in *Ginkgo*. Amer. Nat. **79**: 186-187.

## LINDSAY SHEPHERD OLIVE

B.A., Univ. of North Carolina, 1938; M.A., Ibid., 1940; Ph.D., Ibid., 1942.

Assistant in Botany, Univ. of North Carolina, 1938-1940; Teaching Fellow, 1940-1942; Instructor, 1942-1944.

1940 Observations on the Genus *Gymnosporangium*. (Abst.) U. N. C. Record No. 359: 14-15.

1941 The Germination and Staining of Basidia in *Gymnosporangium*. Stain Tech. 16: 149-153, one pl.

1942 Nuclear Phenomena Involved at Meiosis in *Coleosporium helianthi*. Journ. E. M. Sci. Soc. 58: 43-51, pls. 9, 10.  
(With Leland Shanor) Notes on *Araiospora streptandra*. Mycologia 34: 536-542, 11 figs.

1943 Morphology, Cytology, and Parasitism of *Thekopsora hydrangeae*. Journ. E. M. Sci. Soc. 59: 45-67, pls. 5-12. Abst. in U. N. C. Record No. 383: 32-33. 1942.  
Cytology of Various Basidial Types in the Genus *Septobasidium*. Mycologia 35: 557-572, 3 figs.

1944 New or Rare Heterobasidiomycetes from North Carolina. I. Journ. E. M. Sci. Soc. 60: 17-26, pls. 2-8.  
Spermatial Formation in *Gymnosporangium clavipes*. Mycologia 36: 211-214, two figs.  
Development of the Peritheciun in *Aspergillus Fischeri* Wehmer with a Description of Crozier Formation. Mycologia 36: 266-275, 45 figs.

## JOHN ROBERT RAPEN

B.A., Univ. of North Carolina, 1933; M.A., Ibid., 1936; M.A. and Ph.D., Harvard Univ., 1939.

Assistant in Botany, Univ. of North Carolina, 1931-1934; Teaching Fellow, Ibid., 1934-1936.

1936 Heterothallism and Sterility in *Achlya* and Observations on the Cytology of *Achlya bisexualis*. Journ. E. M. Sci. Soc. 52: 274-289, pls. 22-24. Abst. in U. N. C. Record No. 311: 9.

## DONALD DIRK RITCHIE

B.A., Furman Univ., 1933; B.S., Ibid., 1934; M.A., Univ. of North Carolina, 1937.

Assistant in Botany, Univ. of North Carolina, 1935-1938.

1937 The Morphology of the Peritheciun of *Sordaria fimicola* (Rob.) Ces. and De Not. Journ. E. M. Sci. Soc. 53: 334-342, pls. 28, 29. Abst. in U. N. C. Record No. 323: 10-11.

1938 The Genus *Euglena* at Mountain Lake, Va. (Abst.) Journ. E. M. Sci. Soc. 54: 192-193.

## LELAND SHANOR

B.A., Maryville College, 1935; M.A., Univ. of North Carolina, 1937; Ph.D., Ibid., 1939.  
Assistant in Botany, Univ. of North Carolina, 1935-1936, 1938-1939; Teaching Fellow, Ibid., 1936-1938.

1936 The Production of Mature Perithecia of *Cordyceps militaris* (Linn.) Link in Laboratory Culture. *Journ. E. M. Sci. Soc.* **52**: 99-104, pl. 13.

1937 Observations on the Development and Cytology of the Sexual Organs of *Thraustotheca clavata* (de Bary) Humph. *Journ. E. M. Sci. Soc.* **53**: 119-136, pls. 12, 13. Abst. in *U. N. C. Record No. 323*: 11.

1938 Observations on the Development of a New Species of *Phytophthora*. *Journ. E. M. Sci. Soc.* **54**: 154-162, pls. 15, 16.  
Notes on the Bacterial Disease of the Tubers of the Jerusalem Artichoke, *Helianthus tuberosus*. (Abst.) *Journ. E. M. Sci. Soc.* **54**: 193.

1939 Studies in the Genus *Olpidiopsis* (Cornu) Fischer. I and II. *Journ. E. M. Sci. Soc.* **55**: 167-195, pls. 24, 25 and 4 text figs. Abst. in *U. N. C. Record No. 347*: 13-14.

1940 Studies in the Genus *Olpidiopsis*. III. *Journ. E. M. Sci. Soc.* **56**: 165-176.

## BUDD ELMON SMITH

B.A., Univ. of North Carolina, 1931; M.A., Ibid., 1934; Ph.D., Ibid., 1942.  
Assistant in Botany, Univ. of North Carolina, 1928-1931; Teaching Fellow, 1932-1934; Lecturing Assistant, spring, 1942.

1934 A Taxonomic and Morphological Study of the Genus *Cuscuta*, Dodders, in North Carolina. *Journ. E. M. Sci. Soc.* **50**: 283-302, pls. 27-31.

1942 Some Additions to the Dicotyledonous Flora of South Carolina. (Abst.) *Journ. E. M. Sci. Soc.* **58**: 134-135.

## LAURIE MARGUERITE STEWART

B.A., Appalachian State Teachers' Coll., 1932; M.A., Univ. of North Carolina, 1937.  
Assistant in Botany, Univ. of North Carolina, 1935-1938; Acting Curator of the Herbarium, Ibid., 1938-1942.

1937 Studies in the Life History of *Nitella hyalina* Agardh. *Journ. E. M. Sci. Soc.* **53**: 173-190, pls. 16-19 and one text fig. Abst. in *U. N. C. Record No. 323*: 11.

## HENRY ROLAND TOTTEN

B.A., Univ. of North Carolina, 1913; M.A., Ibid., 1914; Ph.D., Ibid., 1923; Student, Univ. of Paris, Feb.-July, 1919.  
Assistant in Botany, Univ. of North Carolina, 1913-1914; Instructor, Ibid., 1914-1917, 1919-1923; Assistant Professor, 1923-1925; Associate Professor, 1925-1929; Professor, 1929-.

1936 (With W. C. Coker and H. J. Oosting) Dr. Thomas Grant Harbison. *Journ. E. M. Sci. Soc.* **52**: 140-145 (in *Proc. Acad. Sci.*).

1937 (With W. C. Coker) The Trees of the Southeastern States, 2nd ed. 417 pp. The University of North Carolina Press, Chapel Hill.

Notes on *Buckleya* and *Pyrularia* (Buffalo-nut). (Abst.) Journ. E. M. Sci. Soc. **53**: 226.

1945 (With W. C. Coker) The Trees of the Southeastern States, 3rd ed. (419 pp., illus.) The University of North Carolina Press, Chapel Hill.

A Station for *Rhododendron Chapmanii* in Eastern Florida. Proc. Fla. Acad. Sci. **7**, Nos. 2-3.

## MARY WILLIAMS WARD

B.A., Woman's Coll. of Univ. North Carolina, 1937; M.A., Univ. of North Carolina, 1939.

Assistant in Botany, Univ. of North Carolina, 1937-1939.

1939 Observations on a New Species of *Thraustotheca*. Journ. E. M. Sci. Soc. **55**: 346-352, pl. 31.

Observations on *Rhizophlyctis rosea*. Journ. E. M. Sci. Soc. **55**: 353-360, pls. 32, 33. Abst. in U. N. C. Record No. 347: 15.

## ALMA JOSLYN WHIFFEN

B.A., Maryville Coll., 1937; M.A., Univ. of North Carolina, 1939; Ph.D., Ibid., 1941.

Assistant in Botany, Univ. of North Carolina, 1937-1939; Research Assistant, Ibid., 1939-1942.

1939 The Cytology of a New Species of the Plasmodiophoraceae [*Octomyxa Achlyae*]. (Abst.) Journ. E. M. Sci. Soc. **55**: 243. Also in U. N. C. Record No. 347: 15-16.

(With J. N. Couch and Jean Leitner) A New Genus of the Plasmodiophoraceae. Journ. E. M. Sci. Soc. **55**: 399-408, pls. 47, 48.

1941 A New Species of *Nephrochytrium*: *Nephrochytrium aurantium*. Amer. Journ. Bot. **28**: 41-44, 31 figs.

Cellulose Decomposition by the Saprophytic Chytrids. Journ. E. M. Sci. Soc. **57**: 321-330, pl. 8 and 1 text fig. (Abst. on page 202 of same issue.)

1942 Two New Chytrid Genera. Mycologia **34**: 543-557, 52 figs.

A Discussion of Some Species of *Olpidiopsis* and *Pseudolpidium*. Amer. Journ. Bot. **29**: 607-611, 27 figs. Abst. in Journ. E. M. Sci. Soc. **58**: 133.

1943 New Species of *Nowakowskella* and *Blastocladia*. Journ. E. M. Sci. Soc. **59**: 37-43, pls. 2-4.

## \* DEPARTMENT OF CHEMISTRY

## RALPH WALTON BOST

B.A., Newberry Coll., 1923; M.A., Univ. of North Carolina, 1924; Ph.D., Ibid., 1928.

Instructor in Chemistry, Univ. of North Carolina, 1928-1928; Assistant Professor, 1928-1934; Associate Professor, 1934-1937; Professor, 1937-; Head of Department, 1939-.

1934 (With M. W. Conn) A Study of the Action of Sodium Sulfide on Polymethylene Halides and the Formation of "Polythiophanes." Journ. E. M. Sci. Soc. **50**: 182-188.

1935 (With Frank Nicholson) A Color Test for the Identification of Mono-, Di-, and Trinitro Compounds. *Ind. and Eng. Chem., Anal. ed.*, **7**: 190-191. *Abst. in Journ. E. M. Sci. Soc.* **51**: 227.

(With B. O. Cosby) Sulfur Studies. VII. The Preparation and Properties of Some New Thioketones. *Journ. Amer. Chem. Soc.* **57**: 1404-05.

(With Dan Fore, Jr.) The Chemical Composition of the China Berry (*Melia Azedarach*). *Journ. E. M. Sci. Soc.* **51**: 134-142.

(With Frank Nicholson) The Identification of Phenols with 2,4-Dinitrochlorobenzene. *Journ. Amer. Chem. Soc.* **57**: 2368-69.

(With M. W. Conn) n-Propyl Sulfide. *Organic Syntheses* **15**: 72-73.

1936 (With Dudley Williams) Reaction Products of Ethyl Alcohol and Sodium Hydroxide. *Journ. Chem. Phys.* **4**: 251-253.

(With E. W. Constable) Trithioformaldehyde (sym-trithiane). *Organic Syntheses* **16**: 81-83.

1937 (With J. H. Wood) Sulfur Studies. XI. Some Sulfur Derivatives of Benzaldehyde. *Journ. Amer. Chem. Soc.* **59**: 1011-13.

(With J. H. Wood) Sulfur Studies. XII. Thioaldehydes in the Naphthalene and Anthracene Series. *Journ. Amer. Chem. Soc.* **59**: 1721-23.

(With P. H. Latimer) Sulfur Studies. XIII. The Identification of Some Aliphatic Sulfonic Acids. *Journ. Amer. Chem. Soc.* **59**: 2500-01.

(With Dan Fore, Jr.) Sulfur Studies. XIV. Some Derivatives of Certain Higher Mercaptans. *Journ. Amer. Chem. Soc.* **59**: 2557-58.

(With Walker F. Hunter, Jr.) The Destructive Distillation of Peanut Hulls. *Journ. E. M. Sci. Soc.* **53**: 293-297.

1938 [Oxidation Studies I.] The Oxygenation of Diisobutylene at Elevated Pressures. *Science* **88**: 440.

1939 (With Granvil C. Kyker) The Effect of Temperature on the Nitration of p-Cymene. The Synthesis of 6-Nitrocarvacrylamine and Certain Derivatives. *Journ. Amer. Chem. Soc.* **61**: 2469-70.

1940 (With P. H. Latimer) Sulfur Studies. XV. The Synthesis of Alkane Sulfonic Acids and Certain Derivatives. *Journ. Org. Chem.* **5**: 24-28.

(With G. C. Kyker) The Preparation and Properties of 6-Halogenated Carvacrylamines from p-Cymene. *Journ. Amer. Chem. Soc.* **62**: 913-917.

(With B. Berger) Autoxidation and Gum-Forming Tendencies of Certain Hydrocarbons. *Oil and Gas Journ.* **38**, No. 46: 81-86 (Mar. 28).

(With J. E. Everett) Sulfur Studies. XVI. The Synthesis of Certain Higher Alkyl Sulfonium Salts and Related Compounds. *Journ. Amer. Chem. Soc.* **62**: 1752-54.

9-Anthraldehyde; 2-Ethoxy-1-Naphthaldehyde. *Organic Syntheses* **20**: 11-13 (Part B, with J. H. Wood).

1941 (With Charles F. Starnes) Sulfur Studies. XVII. The Synthesis of Sulfathiophene, 2-Sulfanilamidothiophene. *Journ. Amer. Chem. Soc.* **63**: 1885-86.

(With L. B. Lockhart, Jr.) Oxidation Studies. II. The Oxidation of Diisobutylene in the Presence of Potassium Hydroxide at Elevated Temperature and Pressure. *Journ. Amer. Chem. Soc.* **63**: 2790-92.

(With Karl Folkers and Alfred Russell) Explosion Hazard in the Chlorination of Alkyl Isothioureas to Prepare Alkyl Sulfonyl Chlorides. *Journ. Amer. Chem. Soc.* **63**: 3530-32.

1942 (With H. C. Schultz) Sulfur Studies. XVIII. Sulfonium Derivatives of p-Phenylphenacyl Bromide. *Journ. Amer. Chem. Soc.* **64**: 1165-67.

1943 (With E. R. Andrews) Sulfur Studies. XIX. Alkyl Esters of Phenylthiocarbamic Acid. *Journ. Amer. Chem. Soc.* **65**: 900-901.

FRANK KENNETH CAMERON

B.A., Johns Hopkins Univ., 1891; Ph.D., *Ibid.*, 1894.

Professor of Chemistry, Univ. of North Carolina, 1926-.

1934 (With A. S. Wheeler and W. C. Coker) Dr. Francis Preston Venable. *Journ. E. M. Sci. Soc.* **50**: 27-28.

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1935 (With W. H. Baskerville) Ferric Oxide and Aqueous Sulfuric Acid at 25° C. *Journ. Phys. Chem.* **39**: 769-779.

1936 Ferric Sulfate in Aqueous Solutions of Other Sulfates. *Journ. Phys. Chem.* **40**: 689-696.

(With E. O. Huffman) Utilization of Alunite through Fusion with Alkali Sulfides. *Ind. and Eng. Chem.* **28**: 420-422.

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1939 (With David T. Milne) Pulping Whole Cotton with Chlorine. *Ind. and Eng. Chem.* **31**: 1076-1078.

Charles Holmes Herty. *Journ. Amer. Chem. Soc.* **61**: 1619-1624.

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(With W. W. Chen) Cellulose Content of Cotton and Southern Woods. Ind. and Eng. Chem. 34: 224-225.

(With Edward L. Powell) Recovery of Oil from Whole Cotton. Ind. and Eng. Chem. 34: 358-359.

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The Growing and Processing of Whole Cotton. Journ. E. M. Sci. Soc. 59: 1-13.

The following publications resulted from work done under Dr. Cameron while he was Director of the Textile Foundation Research on Fundamental Properties of Textile Wastes.

1938 Fundamental Properties of Textile Wastes. I. Foreword. F. K. Cameron. Textile Research 8: 189-191.

II. Electrophoresis. James E. Magoffin. Textile Research 8: 191-194. Abst. in Journ. E. M. Sci. Soc. 54: 200; also in U. N. C. Record No. 335: 158.

III. Gas Bubbles. Philip H. Burdett. Textile Research 8: 226-230.

IV. Flotation. B. Reid Clanton. Textile Research 8: 270-275.

V. Flotation from Ferric Oxide Sols. B. Reid Clanton. Textile Research 8: 301-304.

VI. Electrophoresis of Platinum and Gold Hydrosols. James E. Magoffin and H. Temple Hatch. Textile Research 8: 305-309.

VII. Electric Charge on Escaping Gas Bubbles. Philip H. Burdett. Textile Research 8: 353-356.

VIII. The Flotation of Colloidal Suspensions. James E. Magoffin and B. Reid Clanton. Textile Research 8: 357-363.

1940 The Influence of Electrolytes on the Ballo-Electric Effect. Philip H. Burdett, James E. Magoffin and F. K. Cameron. Textile Research 10: 149-166. Abst. in U. N. C. Record No. 347: 28. 1939. Note: This and the next three articles are parts of the same series of publications but unnumbered.

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A Tube for Studying Dilute Sediments. Ellington M. Beavers. Textile Research 10: 280-286.

Factors Influencing Flocculation and Precipitation. Theory and Variables. E. M. Beavers, James E. Magoffin and F. K. Cameron. Textile Research 11: 23-29.

1941 IX. Concentration of Sulfur Black Dyes by Froth Flotation. L. C. Surprenant James E. Magoffin and F. K. Cameron. Textile Research 11: 126-138.

Factors Influencing Flocculation and Precipitation. Influence of Initial Particle Size. E. M. Beavers, James E. Magoffin and F. K. Cameron. *Textile Research* 11: 139-158.

**HORACE DOWNS CROCKFORD**

B.S., N. C. State Coll., 1920; M.S., Univ. of North Carolina, 1923; Ph.D., *Ibid.*, 1926.

Teaching Fellow in Chemistry, Univ. of North Carolina, 1921-1922; Instructor, 1922-1926; Assistant Professor, 1926-1930; Associate Professor, 1930-1939; Professor, 1939-; on leave in military service, 1942-.

1934 (With D. J. Brawley) The Solubility of Lead Sulfate in Water and Aqueous Solutions of Sulfuric Acid. *Journ. Amer. Chem. Soc.* 56: 2600.

1935 (With T. B. Douglas) A Note on the Calculation of Heat of Reaction from Values of the Equilibrium Constant at Two Temperatures. *Journ. Amer. Chem. Soc.* 57: 97.

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1936 (With H. O. Farr) The Activity Coefficients of Lead Chloride in Solutions of Barium Nitrate. *Journ. Amer. Chem. Soc.* 58: 87.

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**JAMES TALMAGE DOBBINS**

B.A., Univ. of North Carolina, 1911; M.A., *Ibid.*, 1912; Ph.D., *Ibid.*, 1914.

Associate Professor of Chemistry, Univ. of North Carolina, 1918-1930; Professor, 1930-.

1934 (With J. K. Colehour) The Preparation of Perrhenic Acid. *Journ. Amer. Chem. Soc.* 56: 2054.

(With J. P. Sanders) A Volumetric Method for Determination of Cobalt and Nickel. *Ind. and Eng. Chem.* 6: 459.

1935 (With J. A. Addleston[e]) A Study of the Soda-Alum System II. *Journ. Phys. Chem.* 39: 637.

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1937 (With Laura C. Thomas) A Study of the Soda-Alum System. *Journ. Phys. Chem.* 41: 655.

1939 (With E. C. Markham and H. L. Edwards) Scheme of Qualitative Analysis Involving the Use of Organic Reagents. *Journ. Chem. Educ.* 16: 94.

1943 Semi-Micro Qualitative Analysis. (423 pp.) John Wiley and Sons.

**FLOYD HARRIS EDMISTER**

B.S., Syracuse Univ., 1912; M.S., Louisiana State Coll., 1913; Ph.D., Syracuse Univ., 1918.

Assistant Professor of Chemistry, Univ. of North Carolina, 1923-1925; Associate Professor, 1925-.

1935 Laboratory Experiments in General Chemistry. (69 pp.) Chapel Hill, N. C.

**EDWARD MACK, JR.**

B.A., Princeton Univ., 1913; M.A., Ibid., 1914; Ph.D., Ibid., 1916.

Smith Professor of Chemistry and Head of Department, Univ. of North Carolina, 1935-1939.

1936 The Size and Shape of Molecules. (Abst.) Journ. E. M. Sci. Soc. 52: 168-169; also in U. N. C. Record No. 311: 108.

1937 Why Rubber Stretches. (Abst.) Journ. E. M. Sci. Soc. 53: 239-240; also in U. N. C. Record No. 323: 142.

Remarks on Molecular Structure and van der Waals Forces. Journ. Phys. Chem. 41: 221.

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(With other members of the Department of Chemistry) Laboratory Experiments in General Chemistry. (61 pp.) Chapel Hill, N. C.

**JAMES EDWARD MAGOFFIN**

B.S. (Chem.), Cornell Univ., 1932; Ph.D., Ibid., 1936.

Assistant Professor for Industrial Research, Univ. of North Carolina, 1936-1938; Associate Professor, 1938-1940.

See list of Textile Foundation publications under Frank K. Cameron.

**EDWIN CARLYLE MARKHAM**

B.A., Trinity Coll. (N. C.), 1923; Ph.D., Univ. of Virginia, 1927.

Assistant Professor of Chemistry, Univ. of North Carolina, 1934-1937; Associate Professor, 1937-1941; Professor, 1941-.

1939 (With J. T. Dobbins and H. L. Edwards) Scheme of Qualitative Analysis Involving the Use of Organic Reagents. Journ. Chem. Educ. 16: 94.

The Decomposition of Ammonium-deuterium Chloride. (Abst.) Journ. E. M. Sci. Soc. 55: 252.

1942 (With S. E. Smith) General Chemistry Problems. (190 pp.) Reynal Hitchcock Inc. Houghton-Mifflin Company.

## OSCAR KNEFLER RICE

B.S., Univ. of California, 1924; Ph.D., *Ibid.*, 1926.

Associate Professor of Chemistry, Univ. of North Carolina, 1936-1943; Professor, 1943-.

1937 Internal Volume and the Entropy of Vaporization of Liquids. *Journ. Chem. Phys.* **5**: 353.  
On Transitions in Condensed Systems. *Journ. Chem. Phys.* **5**: 492.

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(With H. C. Campbell) The Explosion of Ethyl Azide in the Presence of Diethyl Ether. *Journ. Chem. Phys.* **7**: 700. Also preprinted for the Symposium on the Kinetics of Homogeneous Gas Reactions, held June 20-22, 1939, by the Division of Physical and Inorganic Chemistry and the University of Wisconsin.  
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1940 Electronic Structure and Chemical Binding. (511 pp.) McGraw-Hill Book Co.  
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1942 The Effect of Intermittent Light on a Chain Reaction with Bimolecular and Unimolecular Chain-Breaking Steps. *Journ. Chem. Phys.* **10**: 440.  
(With W. L. Haden, Jr.) The Chain Photolysis of Acetaldehyde in Intermittent Light. *Journ. Chem. Phys.* **10**: 445.  
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**ALFRED RUSSELL**

B.S., Queen's Coll. (Ireland), 1926; D.Sc., *Ibid.*, 1929.

Associate Professor of Chemistry, Univ. of North Carolina, 1937-1941; Professor, 1941-.

1939 (With S. F. Clark) The Constitution of Natural Tannins. VI. Coloring Matters Derived from 2,5-Dihydroxyacetophenone. *Journ. Amer. Chem. Soc.* **61**: 2651.

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Natural Tanning Materials of the Southeastern United States. A Preliminary Report. Part 1. *Journ. Amer. Leather Chem. Assoc.* **38**: 340.  
(With W. G. Tebbens) Chemical Constitution and the Tanning Effect. I. Simple Esters and Polyesters of Gallic Acid. *Journ. Amer. Chem. Soc.* **64**: 2274.

1943 Natural Tanning Materials of the Southeastern United States. Part II. Domestic Leaf Sumac. *Journ. Amer. Leather Chem. Assoc.* **38**: 1.  
*Conocarpus erecta* (Buttonwood, Zaragoza-Mangrove). A New Domestic Source of Tannin. *Chemurgic Digest* **2**: 4.  
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(With R. L. Kenyon) The Synthesis of Pseudoionone. *Organic Syntheses* **22**: 78.

Natural Tanning Materials of the Southeastern United States. Part III. New Tropical and Subtropical Sources. *Journ. Amer. Leather Chem. Assoc.* **35**: 4.

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Natural Tanning Materials of the Southeastern United States. Part V. *Journ. Amer. Leather Chem. Assoc.* **35**: 355.

(With W. G. Tebbens and W. F. Arey) Chemical Constitution and the Tanning Effect. II. *Journ. Amer. Chem. Soc.* **65**: 1472.

(With L. M. Addison) Vicinal Substituted Resorcinols. Part III. *Journ. Amer. Chem. Soc.* **65**: 2379.

1944 Natural Tanning Materials of the Southeastern United States. Part VI. *Journ. Amer. Leather Chem. Assoc.* **39**: 173.

(With E. A. Kaczka) Fish Poisons from *Ichthyomethia Piscipula*. I. *Journ. Amer. Chem. Soc.* **66**: 548.

#### SHERMAN EVERETT SMITH

B.S., South Dakota School of Mines, 1930; Ph.D., Ohio State Univ., 1935.

Dupont Research Associate in Chemistry, Univ. of North Carolina, 1935-1936; Assistant Professor, 1936-1941; Associate Professor, 1941-1945.

1937 Permeabilities of Cellulosic Films. (Abst.) *Journ. E. M. Sci. Soc.* **53**: 237; also in *U. N. C. Record No. 323*: 140.

1940 (With other members of the Department of Chemistry) Laboratory Experiments in General Chemistry. (310 pp.) Chapel Hill, N. C.

1942 (With E. C. Markham) General Chemistry Problems. (190 pp.) Reynal Hitchcock Inc. Houghton-Mifflin Company.

(With V. L. Simril) Sorption of Water by Cellophane. *Ind. and Eng. Chem.* **34**: 226-230.

1944 (With other members of the Department of Chemistry) Laboratory Experiments in General Chemistry. Revised edition (298 pp.) Chapel Hill, N. C.

#### JONATHAN W. WILLIAMS

B.S., Baldwin-Wallace Coll., 1930; M.S., Northwestern Univ., 1932; Ph.D., *Ibid.*, 1935.

Assistant Professor of Chemistry, Univ. of North Carolina, 1939-41; on leave in government service, 1941-.

1941 (With John A. Krynnitsky) Acetoacetanilide. *Organic Syntheses* **21**: 4-5.

(With John A. Krynnitsky) n-Caproic Anhydride. *Organic Syntheses* **21**: 13-14.

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1942 (With W. T. Rainey, Jr., and R. S. Leopold) Identification of Amides through the Mercury Derivatives. *Journ. Amer. Chem. Soc.* **64**: 1738-1739.

#### CHEMICAL ENGINEERING

##### CARL WILLIAMS BORGmann

B.S., Univ. of Colorado, 1927; Ch.E., *Ibid.*, 1931; Ph.D., Cambridge, 1934.

Assistant Professor of Chemical Engineering, Univ. of North Carolina, 1934-1936.

1935 An Optical Investigation of the Passivity of Iron in Nitric and Chromic Acids. (Abst.) *Journ. E. M. Sci. Soc.* **51**: 248.

##### ALFRED McLaren White

B.S.E., Univ. of Michigan, 1925; M.S., Univ. of California, 1926; Sc.D., Univ. of Michigan, 1928.

Associate Professor of Chemical Engineering, Univ. of North Carolina, 1930-1936.

1935 Pressure Drop and Loading Velocities in Packed Towers. *Trans. Amer. Inst. Chem. Eng.* **31**: 390-408.

New Tower Filling Material. *Science* **81**: 596-597.

#### DEPARTMENT OF GEOLOGY

##### ROBERT OLIVER BLOOMER

B.S., Univ. of Virginia, 1937; M.S., *Ibid.*, 1938; Ph.D., Univ. of North Carolina, 1941.

Assistant in Geology, Univ. of North Carolina, 1938-1939; Instructor, 1939-1941.

1939 (With A. A. Pegau) Geology and Mineral Resources of the Piedmont in the Vicinity of Richmond, Virginia. (Abst.) *Proc. Va. Acad. Sci.* **17**: 58-59.

Nos. 208-213, 1702, 1826, 1831 in Joseph K. Roberts: Catalogue of Topographic and Geologic Maps of Virginia. Dietz Press, Richmond, Va.

Notes on the Petersburg Granite. *Bull. Va. Geol. Surv.* **51-F**: 139-145, 2 pls., 1 fig.

1940 Notes on Varvelike Clay near Buena Vista, Virginia. (Abst.) *Va. Journ. Sci.* **1**: 243 (Proc. Acad. Sci.).

1941 (With R. R. Bloomer) Relations and Age of the Catoctin Formation in Central Virginia. (Abst.) *Bull. Geol. Soc. Amer.* **52**: 1890.

Observations on the Structure of the Blue Ridge in Central Virginia. (Abst.) *Journ. E. M. Sci. Soc.* **57**: 208-209.

Influence of the Core of the Blue Ridge on the Geology of the Buena Vista, Va., Quadrangle. (Abst.) *Va. Journ. Sci.* **2**: 217.

(With Wallace De Witt) Titaniferous Sandstone near Buena Vista, Virginia. *Econ. Geol.* **36**: 745-747, 2 figs.

Geology of the Blue Ridge in the Buena Vista Quadrangle, Virginia. (Abst.) *U. N. C. Record No. 371*: 78-79.

## CARL BARRIER BROWN

B.S., Univ. of North Carolina, 1929; M.A., Univ. of Cincinnati, 1931.

1932 A New Triassic Area in North Carolina. Amer. Journ. Sci., 5th ser., 23: 525-528, 2 figs.

## LANCASTER DEMOREST BURLING

B.S., Univ. of Wisconsin, 1905.

Associate Professor of Geology, Univ. of North Carolina, 1937-1939.

1939 Some Results Obtained with the Prototype of a New Geophysical Instrument. Journ. E. M. Sci. Soc. 55: 252-254. Also in U. N. C. Record No. 347: 187-189.

## JOSEPH ROBINSON CLAIR

B.A., Univ. of Kansas, 1933; M.S., Missouri Sch. of Mines and Metallurgy, 1938; Ph.D., Univ. of North Carolina, 1941.

Instructor in Geology, Univ. of North Carolina, 1939-1941.

1941 Stratigraphy, Structure, and Economic Geology of the Belton Area, Cass County, Missouri. (Abst.) U. N. C. Record No. 371: 79.

1943 Oil and Gas Resources of Cass and Jackson Counties, Missouri. Rept. Mo. Geol. Surv. 27: 1-208, with maps.

## WALLACE DE WITT, JR.

B.A., Univ. of North Carolina, 1943.

Assistant in Geology, Univ. of North Carolina, 1942-1943.

1941 (With R. O. Bloomer) Titaniferous Sandstone near Buena Vista, Virginia. Econ. Geol. 36: 745-747, 2 figs.

## RICHARD ARCHER EDWARDS

B.S., Univ. of Michigan, 1931; M.A., Univ. of Cincinnati, 1934; Ph.D., Univ. of North Carolina, 1938.

Teaching Fellow in Geology, Univ. of North Carolina, 1934-1935; Instructor, 1936-1938, 1939-1940.

1944 Ostracoda from Duplin Marl (Upper Miocene) of North Carolina. Journ. Paleontology 18: 505-528.

## SAMUEL THOMAS EMORY

B.A., Randolph-Macon Coll., 1917; M.A., Ibid., 1918; M.A., Columbia Univ., 1921; Ph.D., Univ. of Chicago, 1939.

Assistant Professor of Geology, Univ. of North Carolina, 1933-1939; Associate Professor, 1939-.

1935 A Canadian Gold Mine. North Car. Educ. 2: 101, 105-106.

1936 Topography and Towns of the Carolina Piedmont. Econ. Geog. 12: 91-97. Abst. in U. N. C. Record No. 311: 53.

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1943 Our Middle American Trade. *Econ. Geog.* 19: 405-417, 6 figs.

1944 North Carolina Ports. *The State* 12, No. 26: 4-5, 18, 1 map.

FRANKLIN CARL ERICKSON

B.A., Clark Univ., 1928; M.A., Ibid., 1930; Ph.D., Ibid., 1935; Exchange Student at Univ. of Zurich, Switzerland, 1932-1933.

Assistant Professor of Geology, Univ. of North Carolina, 1936-1941; Associate Professor, 1941-.

1938 Transhumance in the Land Economy of Schachenthal, Switzerland. *Econ. Geog.* 14: 38-46.

1939 (With W. H. Levitt) Land Utilization of the University Lake Drainage Basin. *Econ. Geog.* 15: 293-302.

Cartographic work for *Elements of Political Geography* by Samuel Van Valkenburg. Prentice-Hall, New York.

1942 Five maps (dealing with Axis Aggression and the N. C. Aircraft Warning System). *Popular Government* 8, No. 4 (Defense Issue).

(With J. C. McCampbell) The Inland Waterway—Can It Be Used for Coastal Shipping? *Popular Government* 8, No. 5: 7, 10-11.

Shifting Centers of Rubber Production. *Popular Government* 8, No. 7: 3-5.

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1944 The Cotton Belt of North Carolina. *Econ. Geog.* 20: 5-8, 1 fig.

1945 The Tobacco Belt of North Carolina. *Econ. Geog.* 21: 58-61, 2 figs.

JOHN WARFIELD HUDDLE

B.S., Northwestern Univ., 1929; Ph.D., Univ. of Indiana, 1934.

Assistant Professor of Geology, Univ. of North Carolina, 1934-1939; Associate Professor, 1939-; on leave, 1944-.

1938 (With G. R. MacCarthy) Shape-sorting of Sand Grains by Wind Action. *Amer. Journ. Sci.*, 5th ser., 86: 64-73, 1 fig. Abst. in *Journ. E. M. Sci. Soc.* 53: 224, 1937; also in *U. N. C. Record* No. 323: 61.

1939 The Paleontologists' View of Species. (Abst.) *Journ. E. M. Sci. Soc.* 55: 251; also in *U. N. C. Record* No. 347: 186.

1940 Notes on the Geologic Section at the Natural Well near Magnolia, N. C. (Abst.) *Journ. E. M. Sci. Soc.* 56: 227-228.

1941 Brown Iron Ore of the Chulafinnee District, Alabama. *Ala. Geol. Surv. Circ.* No. 17.

1942 An Unusual Brown Iron Ore Deposit. (Abst.) *Journ. E. M. Sci. Soc.* 56: 135.

1944 (With J. C. McCampbell) A Laboratory Manual for Beginning Students in General Geology. (90 pp.) Edwards Brothers, Ann Arbor, Mich.

**WILLIAM HAROLD LEWIN**

B.A., Ohio Wesleyan Univ., 1930; M.A., Columbia Univ., 1934; Ph.D., *Ibid.*, 1937.

Assistant Professor of Petrology, Univ. of North Carolina, 1937-1941.

1938 Geology of the Rock Foundation of Grand Coulee Dam, Washington. *Bull. Geol. Soc. Amer.* **49**: 1627-1650, 3 pls., 5 figs.

**W. RAY JOHNSON, JR.**

B.S., Univ. of North Carolina, 1935.

Teaching Fellow in Geology, Univ. of North Carolina, 1937-1939.

1935 (With H. W. Straley, III) An Attempt to Locate the Boundaries of the Durham (N. C.) Triassic Basin with a Magnetometer. *Trans. Amer. Geophys. Union* **16**, pt. 1: 176-181, 5 figs. (Nat. Research Council) Abst. in *Journ. E. M. Sci. Soc.* **51**: 222-223; also in *U. N. C. Record No. 298*: 95-96.

1937 (With G. R. MacCarthy, J. C. McCampbell, and H. W. Straley, III) Tracing a Basic Dike near Chapel Hill, N. C., by Geoelectrical and Geomagnetic Methods. *Amer. Inst. Min. Met. Eng. Contrib.* No. 106, 4 pp., 3 pls. Abst. in *A.I.M.M.E. Yearbook for 1937*: 76-77. 1938.

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(With H. W. Straley, III) Tracing Pegmatite Dikes Geophysically. (Abst.) *Bull. Geol. Soc. Amer.* **49**: 1951.

**WILSON MORROW LAIRD**

B.A., Muskingum Coll., 1936; M.A., Univ. of North Carolina, 1938; Ph.D., Univ. of Cincinnati, 1942.

Teaching Fellow in Geology, Univ. of North Carolina, 1936-1937; Assistant, 1937-1938.

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**THEODORE D. LANCE, JR.**

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**JOHN CALDWELL McCAMPBELL**

B.S., Univ. of North Carolina, 1934; M.S., Vanderbilt Univ., 1936; Ph.D., Univ. of North Carolina, 1944.

Assistant in Geology, Univ. of North Carolina, 1938-1939; Instructor, 1939-1944.

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**GERALD RALEIGH MACCARTHY**

B.A., Cornell Univ., 1921; M.A., Univ. of North Carolina, 1924; Ph.D., *Ibid.*, 1926; Student at Univ. of Poitiers.

Instructor in Geology, Univ. of North Carolina, 1922-1926; Assistant Professor, 1926-1932; Associate Professor, 1932-1940; on leave, 1937-40; Professor, 1940-; on leave in government service, 1942-.

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**MARTHA ELIZABETH NORBURN**

B.A., St. Genevieve of the Pines (Asheville), 1926; M.A., Univ. of Michigan, 1929; Ph.D., Univ. of North Carolina, 1932.

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Instructor in Geology, Univ. of North Carolina, 1938-1939.

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B.S., Syracuse Univ., 1903; M.S., Ibid., 1904; Ph.D., Johns Hopkins Univ., 1906.

Professor of Stratigraphic Geology, Univ. of North Carolina, 1919-; Head of the Department, 1932-.

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**JOE HENRY WATKINS**

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Instructor in Geology, Univ. of North Carolina, 1941-1942.

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**WILLIAM ALEXANDER WHITE**

B.A., Duke Univ., 1930; M.A., Univ. of North Carolina, 1931; M.S., Montana School of Mines, 1934; Ph.D., Univ. of North Carolina, 1938.

Assistant in Geology, Univ. of North Carolina, 1936-1938; Assistant Professor, 1944-.

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## ROBERT L. WRIGLEY, JR.

B.S., Utah State Agricultural Coll., 1934; M.S., Ibid., 1935; Ph.D., Univ. of Chicago 1942.

Assistant Professor of Geography, Univ. of North Carolina, 1942-43.

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## DEPARTMENT OF MATHEMATICS

## REINHOLD BAER

Ph.D., Univ. of Göttingen, 1925.

Assistant Professor of Mathematics, Univ. of North Carolina, 1937-1938.

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## ALFRED THEODOR BRAUER

Ph.D., Univ. of Berlin, 1928.

Assistant Professor of Mathematics, Univ. of North Carolina, 1942-.

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## EDWARD TANKARD BROWNE

B.A., Univ. of Virginia, 1915; M.A., Ibid., 1917; Ph.D., Univ. of Chicago, 1926.

Assistant Professor of Mathematics, Univ. of North Carolina, 1922-1926; Associate Professor, 1926-1932; Professor, 1932-.

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**EDWARD ALEXANDER CAMERON**

B.A., Univ. of North Carolina, 1928; M.A., Ibid., 1929; Ph.D., Ibid., 1936.

Instructor in Mathematics, Univ. of North Carolina, 1928-1937; Assistant Professor, 1937-1941; Associate Professor, 1941-; on leave in government service, 1942-.

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Assistant Professor of Mathematics, Univ. of North Carolina, 1931-.

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**ARCHIBALD HENDERSON**

B.A., Univ. of North Carolina, 1898; M.A., Ibid., 1899; Ph.D., Ibid., 1901; Ph.D., Univ. of Chicago, 1915; Student at Cambridge University, Univ. of Berlin, and The Sorbonne, 1910-1911.

Instructor in Mathematics, Univ. of North Carolina, 1898-1902; Associate Professor, 1903-1908; Professor, 1908-1920; Kenan Professor and Head of the Department, 1920-.

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**THOMAS FELIX HICKERSON**

Ph.B., Univ. of North Carolina, 1904; M.A., *Ibid.*, 1907; B.S., Massachusetts Inst. Technology, 1909.

Instructor in Mathematics, Univ. of North Carolina, 1905-1908; Associate Professor of Civil Engineering, 1910-1920; Professor, 1920-1938; Professor of Applied Mathematics, 1938-.

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B.A., Univ. of North Carolina, 1920; M.A., Ibid., 1921.

Teaching Fellow in Mathematics, Univ. of North Carolina, 1920-1921; Instructor, 1921-1925; Assistant Professor, 1925-1934; Associate Professor, 1934-1941; Professor, 1941-; on leave in military service, 1942-.

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**VINTON ASBURY HOYLE**

B.A., Univ. of North Carolina, 1924; M.A., Ibid., 1925; Ph.D., Princeton Univ., 1930.

Instructor in Mathematics, Univ. of North Carolina, 1925-1927; Assistant Professor, 1930-1936; Associate Professor, 1936-; on leave in military service, 1942-.

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**WITOLD HUREWICZ**

Ph.D., Univ. of Vienna, 1926.

Assistant Professor of Mathematics, Univ. of North Carolina, 1939-1943; on leave 1943-.

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Instructor in Mathematics, Univ. of North Carolina, 1937-1938; Assistant Professor, 1938-1940; on leave, 1940-1941; Associate Professor, 1941-1943.

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**JOHN WAYNE LASLEY, JR.**

B.A., Univ. of North Carolina, 1910; M.A., *Ibid.*, 1911; Ph.D., Univ. of Chicago, 1920.

Fellow in Mathematics, Univ. of North Carolina, 1910-1911; Instructor, *Ibid.*, 1911-1915, 1916-1918; Assistant Professor, 1918-1920; Associate Professor, 1920-1924; Professor, 1924-; Chairman of the Division of Natural Sciences, 1944-.

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**JOE BURTON LINKER**

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Instructor in Mathematics, Univ. of North Carolina, 1919-1922; Assistant Professor, 1924-1927; Associate Professor, 1927-1939; Professor, 1939-; on leave in military service, 1940-1944.

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**ERNEST LLOYD MACKIE**

B.A., Univ. of North Carolina, 1917; M.A., Harvard, 1920; Ph.D., Univ. of Chicago, 1927.

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1940 Review: Introduction to the Theory of Equations by Louis Weisner. *Nat. Math. Mag.* 14: 494-495.

(With V. A. Hoyle) Elementary College Mathematics. (331 pp.) *Ginn and Company*, Boston.

1943 Review: The Calculus by Ettlinger and Porter. *Nat. Math. Mag.* 17: 235.

**RALPH MCCOY TRIMBLE**

C.E., Univ. of Virginia, 1921; M.S., Univ. of North Carolina, 1927.

Instructor in Civil Engineering, Univ. of North Carolina, 1923-1927; Assistant Professor, 1927-1929; Associate Professor, 1929-1938; Associate Professor of Applied Mathematics, 1938-.

1937 (With T. F. Hickerson) Review Questions and Problems (115 in number) in Bruce's Highway Design and Construction. International Textbook Co., Scranton, Pa.

**HENRY WALLMAN**

B.S., Brooklyn Coll., 1933; M.A., Princeton Univ., 1935; Ph.D., Ibid., 1937.

Assistant Professor of Mathematics, Univ. of North Carolina, 1940-1941.

1941 (With Witold Hurewicz) Dimension Theory. (115 pp.) Princeton University Press, Princeton, N. J.

**ARTHUR SIMEON WINSOR**

B.A., Mount Allison Univ., 1914; M.A., Ibid., 1915.

Assistant Professor of Mathematics, Univ. of North Carolina, 1919-1924; Associate Professor, 1924-1943; Professor, 1943-.

1940 Modern Higher Plane Geometry. (118 pp.) Edwards Brothers, Ann Arbor, Mich.

1941 Modern Higher Plane Geometry. (214 pp.) Christopher Publishing House, Boston.

**SCHOOL OF MEDICINE****DEPARTMENT OF ANATOMY****ALWYN JAMES ATKINS**

B.S., Univ. of Chattanooga, 1939; M.A., Univ. of North Carolina, 1942.

1942 A Comparative Study of the Yolk Sac in Amniotes with Particular Reference to the Cytology of Yolk Digesting Cells. (Abst.) U. N. C. Record No. 383: 26.

**WESLEY CRITZ GEORGE**

B.A., Univ. of North Carolina, 1911; M.A., Ibid., 1912; Ph.D., Ibid., 1918.

Instructor in Zoology, Univ. of North Carolina, 1912-1916; Associate Professor of Histology and Embryology, School of Medicine, 1920-1924; Professor, 1924-; Head of Department of Anatomy, 1940-.

1935 The Function and Fate of the Lymphocytes. South. Med. and Surgery 97: 122-125. (Abst.) U. N. C. Record No. 298: 7.

Contact as a Stimulus to Localized Growth. (Abst.) Journ. E. M. Sci. Soc. 51: 215-216; also in U. N. C. Record No. 298: 7-8.

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1941 Comparative Hematology and the Functions of the Leucocytes. *Quart. Rev. Biol.* **16**: 426-439.

Some Early Human Embryos with Particular Reference to the Prochordal Plate. (Abst.) *Journ. E. M. Sci. Soc.* **57**: 197; also in *U. N. C. Record* No. 371: 7.

1942 A Presomite Human Embryo with Chorda Canal and Prochordal Plate. *Carnegie Contrib. Embryol.* **30**: 1-7. (Abst.) *Anat. Rec.* (Supplement) **79**: 22.

1943 (With H. G. Wells and W. A. Perlzwig) Dr. F. H. Swett. *Journ. E. M. Sci. Soc.* **59**: 105 (Proc. N. C. Acad. Sci.).

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## JUNE U. GUNTER

A.B., Univ. North Carolina, 1931. Med. student, Univ. North Carolina Sch. Med., 1931-1934.

Asst. in Anat., Sch. Med., Univ. of North Carolina, 1932-1934.

1934 Anatomy of Venous Valves. (Abst.) *Journ. E. M. Sci. Soc.* **50**: 60; also in *U. N. C. Record* No. 292: 106.

## BENJAMIN FREEMAN KINGSBURY

B.A., Buctel Coll., 1893; M.S., Cornell Univ., 1894; Ph.D., Ibid., 1895; M.D., Univ. of Freiburg, 1903.

Guest Professor of Anatomy, Univ. of North Carolina Medical School, 1941-.

1942 "The Pharyngeal Hypophysis of the Dog." *Anat. Rec.* **82**: 39-57.

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1943 On the So-called Laryngeal Tonsils of Mammals; with Special Reference to their Structure and Development in the Cat. *Amer. Journ. Anat.* **72**: 171-197.

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**FRANK NORMAN LOW**

B.A., Cornell Univ., 1932; Ph.D., *Ibid.*, 1936.

Instructor in Anatomy, Univ. of North Carolina Medical School, 1937-1939; Assistant Professor, 1939-.

1940 (With Walter C. Hilderman) A Case of Hyper-rotation of the Colon. *Anat. Rec.* **77**: 27-30. (Abst.) *U. N. C. Record* No. 347: 7.

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1943 Studies on Peripheral Visual Acuity. *Science* **97**: 586-587.

The Peripheral Visual Acuity of 100 Subjects. *Amer. Journ. Physiol.* **140**: 83-88.

The Measurement of Peripheral Visual Acuity. (Abst.) *Journ. E. M. Sci. Soc.* **59**: 116.

**EDWARD CARL PLISKE**

B.A., Oberlin Coll., 1932; Crane Scholar, Marine Biol. Lab., Woods Hole, Mass., 1932; M.A., Univ. of Minnesota, 1934; Ph.D., *Ibid.*, 1936.

Instructor in Anatomy, Univ. of North Carolina Medical School, 1938-1940; Assistant Professor, 1940-.

1940 Studies on the Influence of the Zona Pellucida in Atresia. *Journ. Morphol.* **67**: 321-349. (Abst., title different) *U. N. C. Record* No. 347: 7-8.

**CHARLES DURWARD VAN CLEAVE**

B.A., Univ. of Colorado, 1925; Ph.D., Chicago, 1928.

Assistant Professor of Anatomy, Univ. of North Carolina Medical School, 1940-1943; Associate Professor, 1943-.

1941 The Inductive Effects of Several Non-Living Organs on Isolated Gastrular Ectoderm. (Abst.) *Anat. Rec.* **79** (Suppl. 2): 60-61.

1943 The Effect of Low Temperature on Neural Induction in Amphibian Ectoderm. (Abst.) *Anat. Rec.* **85**: 53.

**JAMES C. WRENN**

B.A., Duke Univ., 1935; Student, Univ. of North Carolina Medical School, 1935-1937.

1937 A Case of Retrocaval Ureter. *Anat. Rec.* **68**: 389-392.

## DEPARTMENT OF BACTERIOLOGY

## HERMAN CHARLES MASON

B.S., Univ. of Chicago, 1932; M.S., Univ. of Illinois, 1936; Ph.D., *Ibid.*, 1939

Associate Professor of Bacteriology, Univ. of North Carolina, 1942-1944.

1943 (With Deane Vance and Thornton Scott) Inability to Pass Primary Atypical Pneumonia to Human Volunteers. *Science* **98**: 412-413.1944 (With Ellsworth Twyble) Hemagglutination By Products of Influenza Virus Using Infected Mouse-Lung and Chick-Embryo as Source of Virus. *Journ. Immunology* **49**: 73-86.

## DEPARTMENT OF BIOLOGICAL CHEMISTRY

## JAMES CLARENCE ANDREWS

B.S., Univ. of Iowa, 1915; Ph.D., Columbia Univ., 1918.

Professor of Biological Chemistry and Head of the Department, Univ. of North Carolina Medical School, 1937-.

1937 (With Kathleen C. Andrews) Metabolic Production of Sulfate from Cyclic Sulfonic Acids Related to Cystine and Methionine. (Abst.) *U. N. C. Record* No. 323: 13.The Hydrolysis of Hair. (Abst.) *U. N. C. No.* 323: 12.1938 Reduction of Certain Sulfur Compounds to Hydrogen Sulfide by the Intestinal Microorganisms of the Dog. *Journ. Biol. Chem.* **122**: 687-692. (Abst.) *U. N. C. Record* No. 323: 12.Mechanism of Acid-Base Control by Urine Excretion. (Abst.) *Journ. E. M. Sci. Soc.* **54**: 203; also in *U. N. C. Record* No. 335: 160-161.

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(With W. E. Cornatzer) The Use of Ficin as an Anthelmintic. *Proc. Amer. Soc. Biol. Chem.* **35**: iv-v.

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(With R. E. Brooks) A Study of a Cystinuric Family. *Journ. Urology* **47**: 171-173.

(With W. E. Cornatzer and A. B. Sample) The Extraction of Proteins from Aqueous Solution by Means of Emulsification with Chloroform. *Journ. Lab. and Clin. Med.* **27**: 941-948. (Abst.) *Fed. Proc. Amer. Soc. Exp. Biol.* **1**: 98; also in *U. N. C. Record* No. 383: 28.

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(With Bailey D. Webb) The Effect of Hookworm Damage of Levels of Quinine Attained in Blood and Urine of Dogs Following Single Doses of Quinine Sulfate. *Journ. Pharm. and Exp. Therap.* **75**: 191-195. (Abst.) *U. N. C. Record* No. 383: 29.

(With Carl E. Anderson) The Enzymatic Conversion of Quinine in Vitro by Tissues of the Rat. (Abst.) *Fed. Proc. Amer. Soc. Exp. Biol.* **1**: 97.

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The Chemistry and Metabolism of the Compounds of Sulfur. *Ann. Rev. Biochem.* **12**: 115-134.

(With Carl E. Anderson) The Absorption of Quinine Sulfate and Quinine Dihydrochloride from Isolated Intestinal Loops of Dogs. *Journ. Pharm. and Exp. Therap.* **78**: 346-351.

(With W. E. Cornatzer) The Dissociation Constants and Optical Activity of Quitenine. *Journ. E. M. Sci. Soc.* **59**: 177-180.

(With W. E. Cornatzer) Effect of Hemorrhagic Anemia on Quinine Excretion. (Abst.) *Fed. Proc. Amer. Soc. Exp. Biol.* **2**: 57.

(With W. E. Cornatzer) Effect of Liver Damage on Quinine Excretion. (Abst.) *Fed. Proc. Amer. Soc. Exp. Biol.* **2**: 57.

1944 Why Teach Nutrition? *The High School Journal* **27**: 6-9.

(With W. E. Cornatzer and Mildred M. McEwen) Schizonticidal Tests on *Rauwolfia heterophylla* and Some Other Proposed Antimalarial Plants. *Journ. E. M. Sci. Soc.* **60**: 167-170.

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## CARL E. ANDERSON

B.S., Univ. of Connecticut, 1935; Ph.D., Univ. of North Carolina, 1943.

1943 (With W. E. Cornatzer and James C. Andrews) The Rôle of the Liver in the Metabolic Destruction of Quinine. *Journ. Pharm. and Exp. Therap.* **79**: 62-69.

(See also under James C. Andrews.)

## WILLIAM EUGENE CORNATZER

B.S., Wake Forest Coll., 1939; M.S., Univ. of North Carolina, 1941; Ph.D., *Ibid.*, 1944.

Fels Assistant, Biological Chemistry, Univ. of North Carolina, 1939-1940; Graduate Assistant, 1940-1943; Fels Research Fellow, 1943-.

1941 (With J. C. Andrews) The Use of Ficin as an Anthelmintic. (Abst.) *Proc. Amer. Soc. Biol. Chem.* **35**: iv-v.

1942 (With J. C. Andrews) Some Properties of Ficin. *Journ. Pharm. and Exp. Therap.* **74**: 129-133. (Abst.) *U. N. C. Record No. 383*: 28.

(With J. C. Andrews and A. B. Sample) The Extraction of Proteins from Aqueous Solution by Means of Emulsification with Chloroform. *Journ. Lab. and Clin. Med.* **27**: 941-948. (Abst.) *Fed. Proc. Amer. Soc. Exp. Biol.* **1**: 98; also in *U. N. C. Record No. 383*: 28.

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(With James C. Andrews) The Effect of Acid and Alkali on the Absorption and Metabolism of Quinine. *Journ. Pharm. and Exp. Therap.* **82**: 261-265.

## GRANVIL CHARLES KYKER

B.S., Carson-Newman Coll., 1932; Ph.D., Univ. of North Carolina, 1938.

Instructor in Biological Chemistry, Univ. of North Carolina, 1937-1938; Assistant Professor, 1938-1943; Associate Professor, 1943-.

1938 Separation of Proteins from Aqueous Solutions by Extraction with Chloroform. (Abst.) *U. N. C. Record No. 335*: 9.

1939 (With R. W. Bost) The Effect of Temperature on the Nitration of p-Cymene. The Synthesis of 6-Nitrocarvacrylamine and Certain Derivatives. *Journ. Amer. Chem. Soc.* **61**: 2469-2470.

(With James C. Andrews) The Determination of Quinine in the Blood. (Abst.) *Proc. Amer. Soc. Biol. Chem.* **33**: lviii-lix.

1940 (With R. W. Bost) The Preparation and Properties of 6-Halogenated Carvacrylamines from p-Cymene. *Journ. Amer. Chem. Soc.* **62**: 913-917. Abst. in U. N. C. Record No. 335: 17-18. 1938.

(With Bailey D. Webb and James C. Andrews) The Nephelometric Determination of Quinine in Small Amounts by Means of the Photoelectric Colorimeter. (Abst.) *Proc. Amer. Soc. Biol. Chem.* **34**: lvii.

1941 (With Bailey D. Webb and James C. Andrews) The Estimation of Small Amounts of Quinine in Blood and Other Biological Material. *Journ. Biol. Chem.* **139**: 551-567. Abst. in U. N. C. Record No. 371: 10-11.

(With Bailey D. Webb) The Application of the Photelometric Method for the Determination of Quinine in Blood and Urine. (Abst.) *Proc. Amer. Soc. Biol. Chem.* **35**: lxxiii.

1942 The Response of the Cinchonas and Other Bases to the Photelometric Estimation of Quinine. (Abst.) *Fed. Proc. Amer. Soc. Exp. Biol.* **1**: 120.

1943 Quinine Administration during Progressive Vitamin B-Complex Deficiency. (Abst.) *Fed. Proc. Amer. Soc. Exp. Biol.* **2**: 65.

(With W. E. Cornatzer) Photodecomposition of Quinine. (Abst.) *Fed. Proc. Amer. Soc. Exp. Biol.* **2**: 65.

(With Dorothy Plonk) Quinine Analytical Interference Studies. (Abst.) *Fed. Proc. Amer. Soc. Exp. Biol.* **2**: 65.

1944 (With Mildred McEwen) Quinine and Vitamin B-Complex Deficiency. (Abst.) *Fed. Proc. Amer. Soc. Exp. Biol.* **3**: 61.

1945 (With Dorothy Plonk Lewis) The Micro Determination of Certain Alkaloids and Other Bases by Photometric Turbidimetry. *Journ. Biol. Chem.* **157**: 707-716

#### CLINICAL

##### WALTER REECE BERRYHILL

B.A., Univ. of North Carolina, 1921; M.D., Harvard, 1927.

University Physician, Univ. of North Carolina, 1933-1941; Associate Professor of Medicine, 1935-1941; Professor of Medicine and Dean of the Medical School, 1941-.

1943 (With E. McG. Hedgpeth and others) Primary Atypical Pneumonia, Etiology Unknown. *N. C. Med. Journ.* **4**: 421-430.

1944 (With E. McG. Hedgpeth and others) Spontaneous Pneumothorax: Diagnosis and Prognosis. *N. C. Med. Journ.* **5**: 48-52.

Postwar Planning in North Carolina. *The Crippled Child* **22**: 78-79, 90.

## EDWARD McGOWAN HEDGPETH

B.A., Univ. of North Carolina, 1927; M.D., Univ. of Pennsylvania, 1931.

Associate University Physician, Univ. of North Carolina, 1937-1941; University Physician, 1941-.

1944 (With W. R. Berryhill and others) Spontaneous Pneumothorax: Diagnosis and Prognosis. *N. C. Med. Journ.* 5: 48-52.

Penicillin. *Southern Med. and Surg.* 106: 238-242.

## ISAAC HALL MANNING, JR.

A.B., University of North Carolina, 1931; M.D., Harvard, 1935.

Instructor in Medicine, Univ. of North Carolina, 1940-.

1943 (With D. F. Milam) Medical and Nutritional Survey of 800 N Y A Youths; Experience at National Youth Administration Work Center in Physical Rehabilitation of Youths of Draft Age. *South. Med. Journ.* 36: 373-380.

1944 (With D. F. Milam) Vitamin C Nutrition under Camp Conditions. *North Carolina Med. Journ.* 5: 41-43.

## DEPARTMENT OF PATHOLOGY

## CLARK EDWARD BROWN

B.S., St. Lawrence Univ., 1926; M.D., Univ. of Pennsylvania, 1930.

Assistant Professor of Pathology, Univ. of North Carolina, and Pathologist, Watts Hospital, 1940-1942; Associate Professor, 1942-; on leave in naval service since Jan. 1943.

1943 (With George L. Crane) Bilateral Cortical Necrosis of the Kidneys Following Severe Burns. *Journ. Amer. Med. Assoc.* 123: 871-873.

Dietary Ulcers of the Esophagus of the Rat. *Amer. Journ. Path.* 19: 785-795.

## JAMES BELL BULLITT

B.A., Washington and Lee, 1894; M.A., Ibid., 1895; M.D., Univ. of Virginia, 1897.

Professor of Pathology and Head of the Department, Univ. of North Carolina, 1913-.

1934 (With J. T. Dobbins and Otto Stuhlman, Jr.) Dr. James Munsie Bell. *Journ. E. M. Sci. Soc.* 50: 28-29 (Proceedings of Academy of Science).

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1941 Resolution on Harvey Park Barret. *Journ. E. M. Sci. Soc.* 57: 182-183 (Proceedings).

1943 (With George L. Carrington) Pregnancy after Tubal Sterilization. *Amer. Journ. Obs. and Gynecol.* 45: 892-896.

## RUSSELL LOWELL HOLMAN

B.A., Washington Univ., 1927; M.D., Vanderbilt Univ., 1931.

Assistant Professor of Pathology, Univ. of North Carolina, 1937-1939; Associate Professor, 1939-1942; Professor, 1942-.

1938 (With Edward B. Self) The Ability of Lymph to Maintain Viability in "Devascularized" Lymph Nodes. *Amer. Journ. Path.* **14**: 463-472.

Studies on the Chemical Composition and Functional Significance of Mammalian Lymph. *Science* **88**: 478.

1939 Complete Anuria Due to Blockage of Renal Tubules by Protein Casts in a Case of Multiple Myeloma. *Arch. Path.* **27**: 748-752.

1940 (With J. Gilmer Mebane) The Influence of Nitrogen Retention upon the Regeneration of Plasma Proteins. *Journ. Exp. Med.* **71**: 299-304.

1941 Acute Necrotizing Arteritis, Aortitis, and Auriculitis Following Uranium Nitrate Injury in Dogs with Altered Plasma Proteins. *Amer. Journ. Path.* **17**: 359-375.

1942 (With Willard C. Hewitt) Experimental Necrotizing Arteritis II. Mercuric Chloride as Effective as Uranium Nitrate in its Production. *Proc. Soc. Exp. Biol. and Med.* **49**: 58-62.

(With G. L. Donnelly) The Stimulating Influence of Sodium Citrate on Cellular Regeneration and Repair in the Kidney Injured by Uranium Nitrate. *Journ. Pharm. and Exp. Therap.* **75**: 11-17.

(With G. L. Donnelly) Hypoproteinemia as Protection against Mercuric Chloride Injury in Dogs. *Journ. Exp. Med.* **76**: 511-518.

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Experimental Necrotizing Arteritis in Dogs IV. Alteration of the Blood Plasma Proteins not Essential. *Amer. Journ. Path.* **19**: 159-167.

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1944 Renal Injury and Lymphatic Atrophy. Lymphopenia and Atrophy of Lymphatic Tissue Associated with Acute Renal Insufficiency in Dogs. *Arch. Path.* **37**: 124-130.

(With W. A. Douglass) Studies on Heavy Metal Poisoning: I. The Use of Natural Radioactivity for Tracer Studies on Uranium. *Proc. Soc. Exp. Biol. and Med.* **57**: 72-75.

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## RANDOLPH TUCKER SHIELDS

A.B., Washington and Lee Univ., 1898; M.D., Univ. College of Medicine, Richmond, Va., 1901.

Acting-Associate Professor of Pathology, Univ. of North Carolina, 1943-44.

1944 Medicine in China. *South. Med. and Surgery* 108: 113-116.

## DEPARTMENT OF PHARMACOLOGY

## GRANT LESTER DONNELLY

A.B., Univ. of N. C., 1927; M.D., Duke Univ., 1931.

Assistant Professor of Pharmacology, Univ. of North Carolina, 1931-1933; Associate Professor, 1933-1943.

1938 Sulfanilamide—Pharmacology and Therapeutics. *Trans. Med. Soc. State of North Carolina* 85: 501-509.

1939 (With Wm. deB. MacNider) Value of Omentopexy in Establishing an Adventitious Circulation in the Normal Kidney. *Proc. Soc. Exper. Biol. and Med.* 40: 271-272.

1942 Permanent Devocalization of Dogs by Removal of Both True and False Vocal Cords. *Proc. Soc. Exper. Biol. and Med.* 50: 79-80.

(With R. L. Holman) The Stimulating Influence of Sodium Citrate on Cellular Regeneration and Repair in the Kidney Injured by Uranium Nitrate. *Journ. Pharm. and Exper. Therap.* 75: 11-17.

1943 (With R. L. Holman) Stimulating Influence of Sodium Citrate on Cellular Repair in Kidney Injured by Uranium Nitrate. *North Carolina Med. Journ.* 4: 11-12.

## WILLIAM DEBERNIERE MACNIDER

M.D., Univ. of North Carolina, 1903; Student at Chicago and Western Reserve Universities.

Assistant in Biol., Univ. of North Carolina, 1899-1900; Asst. in Anatomy, 1900-02; Demonstrator of Clinical Pathology, 1902-05; Professor of Pharmacology and Bacteriology, 1905-11; Professor of Pharmacology, 1911-18; Kenan Professor, 1918-1924; Kenan Research Professor, 1924-.

1935 Individualism and Freedom. *Southern. Med. and Surg.* 97: 618-619.

A Consideration of the Susceptibility and the Resistance of Tissues to the General Anesthetics. *The Diplomate* 7: 11-16.

The Acquired Resistance of Liver Cells to the Toxic Action of Uranium Nitrate. *Proc. Soc. Exper. Biol. and Med.* 32: 791-793.

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##### HARRY DAVIS BRUNER

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##### JOHN HOWARD FERGUSON

B.A., Cape Town, 1921; B.A., Oxford (Honour School), 1925; M.D., Harvard, 1928; M.A., Oxford, 1931; L.M.S.S.A., London, 1931.

Professor of Physiology and Head of Department, Univ. of North Carolina Medical School, 1943-.

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## SCHOOL OF PHARMACY

## JOHN GROVER BEARD

Ph.G., Univ. of North Carolina, 1909.

Instructor in Pharmacy, Univ. of North Carolina, 1909-1913; Assistant Professor, 1913-1917; Associate Professor, 1917-1919; Professor, 1919-; Dean of the School of Pharmacy, 1931-.

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**DEPARTMENT OF PHYSICS****ERNEST SCOTT BARR**

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Assistant in Physics, Univ. of North Carolina, 1933-1934; Teaching Fellow, 1934-1935; Instructor, 1935-1936.

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**CLIFFORD K. BECK**

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**THOMAS N. GAUTIER**

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**CREIGHTON CLINTON JONES**

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**RUSSELL LYDDANE**

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**HERMAN MANLEY PARKER**

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## EARLE KEITH PLYLER

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**PAUL EDMONDSON SHEARIN**

B.A., Univ. of North Carolina, 1929; M.A., *Ibid.*, 1930; Ph.D., Ohio State Univ., 1934.

Assistant in Physics, Univ. of North Carolina, 1929-1930; Instructor, 1930-1931, 1936-1939; Assistant Professor, 1939-1943; Associate Professor, 1943-.

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**OTTO STUHLMAN, JR**

B.A., Univ. of Cincinnati, 1907; M.A., Univ. of Illinois, 1909; Ph.D., Princeton Univ., 1911.

Associate Professor of Physics, Univ. of North Carolina, 1920-1924; Head of Department, 1928-1934; Professor, 1924-.

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**DEPARTMENT OF PSYCHOLOGY****ENGLISH BAGBY**

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#### JAMES RUEY PATRICK

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**DOROTHY RETHLINGSHAFER**

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**ROBERT JAMES WHERRY**

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#### DELOS DONALD WICKENS

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Instructor in Psychology, Univ. of North Carolina, 1933-1937.

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#### SCHOOL OF PUBLIC HEALTH

##### HERMAN GLENN BAITY

B.A., Univ. of North Carolina, 1917; B.S., Ibid., 1922; M.S., Harvard University, 1925; Sc.D., Ibid., 1928.

Assistant in Physics, Univ. of North Carolina, 1915-1917; Instructor in Mathematics, 1920-1922; Associate Professor of Sanitary and Municipal Engineering, 1926-1929; Professor, 1929-; Dean of the School of Engineering, 1931-1936.

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#### MARGARET BLEE

Certificate in Public Health Nursing, Vanderbilt Univ., 1933; B.S., Missouri Univ., 1934; M. Ed. in Adult Education, Mills Coll., 1941.

Assistant Professor of Maternal and Child Hygiene, Univ. of North Carolina, 1941-.

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#### STERLING BRACKETT

B.A., Univ. of Minnesota, 1935; M.A., Ibid., 1937; Ph.D., Univ. of Wisconsin, 1940.

Instructor in Public Health, Univ. of North Carolina, 1940-1941; Assistant Professor, 1941-1943.

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## HAROLD W. BROWN

B.A., Kalamazoo Coll., 1924; M.S., Kansas State Coll., 1925; Sc.D., Johns Hopkins Univ., 1928; M.D., Vanderbilt Univ. Med. School, 1933; Letter of Credit, London School of Hygiene and Tropical Medicine, 1935; Dr. P. H., Harvard Univ., 1936.

Professor of Public Health, Univ. of North Carolina, 1937-1943; Dean, School of Public Health, 1941-1943.

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## WILLIAM LEROY FLEMING

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Research Professor of Syphilology, Univ. of North Carolina, 1939-.

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## HAROLD BENEDICT GOTAAAS

B.S., Univ. of South Dakota, 1928; M.S., Iowa State Coll., 1930; M.S., Harvard Univ., 1937; Sc.D., Ibid., 1942.

Assistant Professor of Sanitary Engineering, Univ. of North Carolina, 1937-1940; Associate Professor, 1940-1941; Professor, 1941-; on leave in government service, 1942-.

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JOHN J. HANLON

B.S., Massachusetts Inst. Technology, 1933; M.S., Ibid., 1934; M.D., Wayne Univ. Coll. of Medicine, 1940; M.P.H., Johns Hopkins School of Hygiene, 1942.

Acting Associate Professor of Public Health Administration, Univ. of North Carolina, 1943-1944.

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B.S., Massachusetts Inst. Technology, 1933; M.S., Ibid., 1934; Ph.D., Ibid., 1937.

Assistant Professor of Sanitary Engineering, Univ. of North Carolina, 1942-1944.

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JOHN E. LARSH, JR.

B.A., Univ. of Illinois, 1939; M.S., Ibid., 1940; Sc.D., Johns Hopkins School of Hygiene, 1943.

Assistant Professor of Public Health, Univ. of North Carolina, 1943-.

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**ROBERT BARRETT LAWSON**

B.A., Harvard Univ., 1932; M.D., Harvard Medical School, 1936.

Professor of Pediatrics, School of Public Health, Univ. of North Carolina, 1940-1942.

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**DANIEL FRANK MILAM**

B.A., Vanderbilt Univ., 1916; M.D., Univ. of Chicago, 1924; M.P.H., Johns Hopkins School of Hygiene, 1929.

Professor of Nutrition, Univ. of North Carolina, 1939-.

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## LUCY S. MORGAN

B.A., Univ. of Tennessee, 1922; M.A., Columbia Univ., 1929; M.S., Univ. of Tennessee, 1932; Ph.D., Yale Univ., 1938.

Professor of Health Education, Univ. of North Carolina, 1942-.

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## JOHN WILLIAM ROY NORTON

B.A., Trinity Coll., 1920; M.D., Vanderbilt Univ., 1928; M.P.H. Harvard Univ., 1936.

Professor of Public Health Administration, Univ. of North Carolina, 1938-; on leave in military service, Nov. 1940-.

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## EMANUEL WALETZKY

B.A., Univ. of Wisconsin, 1934; M.A., Ibid., 1934; Ph.D., Ibid., 1938.

Instructor in Public Health, Univ. of North Carolina, 1941-1943.

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## DEPARTMENT OF ZOOLOGY

## ANNIE DOROTHY AYCOCK

B.A., Atlantic Christian Coll., 1937; M.A., Univ. of North Carolina, 1941.

Assistant in Zoology, Univ. of North Carolina, 1938-1939.

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## CHARLES DALE BEERS

B.A., Univ. of North Carolina, 1921; M.A., Ibid., 1922; Ph.D., Johns Hopkins Univ., 1925.

Instructor in Zoology, Univ. of North Carolina, 1921-1922; Assistant Professor, 1927-1929; Associate Professor, 1929-1937; Professor, 1937-.

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#### MILDRED IRENE BOLIEK

B.A., Woman's Coll. of U. N. C., 1929; M.A., Univ. of North Carolina, 1933; Ph.D., *Ibid.*, 1936.

Assistant in Zoology, Univ. of North Carolina, 1932-1935.

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#### WILLIAM JONES BOWEN

B.A., Univ. of Buffalo, 1934; M.A., *Ibid.*, 1935; Ph.D., Johns Hopkins Univ., 1938.

Instructor in Zoology, Univ. of North Carolina, 1940-41; Assistant Professor, 1941-1942, through December, 1942.

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#### ROBERT ERVIN COKER

B.S., Univ. of North Carolina, 1896; M.S., *Ibid.*, 1897; Ph.D., Johns Hopkins Univ., 1906.

Assistant in Zoology, Univ. of North Carolina, 1895-1897; Instructor in Biology, summers of 1897 and 1899; Professor of Zoology, 1922-1939; Head of Department, 1935-; Chairman, Division of Natural Sciences, 1935-1944; Kenan Professor, 1939-.

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DONALD EUGENE COPELAND

B.A., Univ. of Rochester, 1935; M.A., Amherst Coll., 1937; Ph.D., Harvard, 1941.

Instructor in Zoölogy, Univ. of North Carolina, 1941-1942.

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DONALD PAUL COSTELLO

B.A., College of the City of Detroit, 1930; Ph.D., Univ. of Pennsylvania, 1934.

Assistant Professor of Zoölogy, Univ. of North Carolina, 1935-1941; Associate Professor, 1941-1943; Professor, 1943-.

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**JAMES WORKMAN CULBERTSON**

B.S., Furman Univ., 1932; M.A., Univ. of North Carolina, 1934.

Teaching Fellow in Zoölogy, Univ. of North Carolina, 1932-1934.

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**EZDA MAY DEVINEY**

B.A., Woman's Coll. of Univ. of North Carolina, 1919; M.S., Univ. of Chicago, 1924; Ph.D., Univ. of North Carolina, 1934.

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**WILLIAM LOUIS ENGELS**

B.S., Univ. of Notre Dame, 1930; Ph.D., Univ. of California, 1937.

Instructor in Zoology, Univ. of North Carolina, 1937-1939; Assistant Professor, 1939-1941; Associate Professor, 1941-; on leave in military service, 1942-.

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**JOHN ALBERT FINCHER**

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**GORDON HERSCHEL TUCKER**

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JOSEPH MANSON VALENTINE

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